Regulation of the Actin Binding Activity of Basic Calponin and Its Implication in Cytokinesis

Thesis

REGULATION OF THE ACTIN BINDING ACTIVITY OF BASIC CALPONIN AND ITS IMPLICATION IN CYTOKINESIS

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To my family

ABSTRACT

Calponins (CaPs) are actin-binding proteins that stabilize actin filaments. Mammals express three genetic isoforms in a tissue-specific manner. CaPs share an N-terminal calponin-homology (CH) domain followed by short tandem sequence repeats termed calponin-like (CLIK[23]) repeats. The extreme C-termini of CaPs contain isoform-specific tail sequences. Basic calponin (h1CaP) was the first isoform to be discovered 22 years ago in an attempt to identify troponin-like proteins in smooth muscle. H1CaP is mainly expressed in smooth muscle cells and was shown to regulate contraction by inhibiting actin-activated myosin ATPase activity. Several mechanisms have been proposed for the regulation of the actin-binding activity of h1CaP. One mechanism is based on phosphorylation. H1CaP phosphorylation inhibits its interaction with actin filaments and releases the block on the myosin ATPase activity *in vitro*. However, *in vivo* it has not been unequivocally demonstrated whether h1CaP is phosphorylated or not. The binding of extracellular regulated kinase (ERK) but not phosphorylation was proposed as another regulatory mechanism, but the physiological relevance of this interaction remains elusive. Therefore, the scope of this thesis was to reveal the mechanisms that regulate the actin-binding activity of h1CaP. First, the contribution of the major h1CaP phosphorylated sites to its actin-binding activity was re-evaluated. A mutant h1CaP mimicking its phosphorylated state behaved, however, similar to wild-type h1CaP. Thus, another regulatory mechanism was explored. It has previously been shown that the C-terminal tails regulate the actin-binding activity of all three CaP isoforms. Also, electron microscopy images of purified h1CaP show a flexible molecule that can adopt both an extended and a more compact conformation, and it was recently shown that the CH domain does not mediate the actin binding-activity of h1CaP. In light of the flexibility of h1CaP and the demonstrated regulatory

role of the C-terminal tail, a possible regulatory mechanism could thus involve an intramolecular interaction between different domains. Therefore, the contribution of the C-terminal tail and the CH domain of h1CaP to its actin-binding activity was investigated in cells. The results suggest that the h1CaP CH domain blocks the inhibitory effects of its tail, which could be mediated by a CH domain–tail interaction. Therefore, the interaction between the CH domain and the tail was modelled and the plausibility of their interaction was predicted *in silico*. Furthermore, the interaction between these two domains was demonstrated *in vitro* using recombinant proteins. The proximity between the CH domain and the tail was also analyzed using FRET imaging in live cells, and the region encompassing helix A and loop 1 of the CH domain was determined to be the binding region for the tail. These results suggest that the binding of h1CaP to actin filaments is modulated by an interaction between the h1CaP N-terminal CH domain and its C-terminal tail.

One aspect of the potential *in-vivo* functions of h1CaP that has been neglected so far relates to its actin-binding activity during cell division. It has previously been reported that overexpression of CaP reduces cell proliferation and increases the number of bi-nucleated cells. This suggests that CaPs have a role during mitosis. Thus, the localization of CaPs during cell division was investigated and the data presented in this study demonstrate the specific accumulation of CaP at the ingression furrow and the contractile ring, suggesting a role in cytokinesis. Furthermore, overexpression of h1CaP mutants led to cell division defects. In summary, this study has revealed a novel regulatory mechanism that controls the actin-binding activity of h1CaP, which is mediated by the intramolecular interaction of its CH domain and its C-terminal tail.

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CHAPTER 1: INTRODUCTION

1.1 Cytoskeleton

The idea of the cytoskeleton dates back to the beginning of the 20th century. In 1929, Parker suggested the presence of fibrils that might have a supportive role for the cytoplasm (Parker, 1929). A similar idea was already proposed in 1916 from the observation of egg cells from the gastropod *Crepidula plana* recovering their original shape after being exposed to a stress (Conklin, 1916). However, it was not until 1940, with the advance of electron microscopy and protein biochemistry that a clearer picture of the components of the cytoskeleton started to emerge. Electron microscopy together with the advance of thin-sectioning technology led to the distinction of two types of cytoskeletal filaments, the actin filaments (F-actin), also called microfilaments, and microtubules (Hanson and Huxley, 1953; Huxley, 1953; Pease, 1963; Phillips, 1966). While actin filaments have been discovered for the first time in studies of striated muscle, microtubules were discovered as the main structures of cilia and flagella. The dawn of monoclonal antibody production (circa 1970) allowed the creation of highly specific reagents that significantly advanced the ability to identify and detect the intracellular distribution of actin and microtubule filaments (Fuller et al., 1975; Lazarides and Weber, 1974; Weber et al., 1975). Indeed, this biotechnology led to the discovery of a third type of cytoskeletal filament termed intermediate filaments (Bignami et al., 1972; Fuchs and Green, 1978; Lazarides and Hubbard, 1976).

Today, it is well established that these filaments can be distinguished on the basis of their diameter, type of protein subunit, arrangement of the subunits, and their cellular functions. The cytoskeleton is involved in diverse functions, such as cell

migration, cell shape regulation, cell division, transport of organelles and molecules, cell-cell and cell-substrate attachment, anchorage of metabolic pathways to a specific location within the cytoplasm, and integration of molecular signalling processes.

Microtubules are present in all eukaryotes. They are the structural components of cilia and flagella in eukaryotic cells and they contribute to the transport of vesicles, to cell shape, to the positioning of the nucleus and organelles, and to chromosome segregation during cell division. Microtubules are made of repeating globular α/β-tubulin heterodimers that are arranged in a cylindrical array of 24 nm diameter. α- and β- tubulin are 50% identical at the amino-acid level, and each subunit has a molecular weight of ~50-kDa (Burns, 1991). Microtubules are highly dynamic, and polymerize or depolymerize according to the needs of the cell. The dynamics of microtubules is regulated by binding and hydrolysis of guanosine triphosphate (GTP) by β-tubulin subunits (Desai and Mitchison, 1997), and the interactions with a large number of microtubule-associated proteins. The two ends of the microtubule have different rates of assembly due to the head to tail association of the α/β-heterodimer (Amos and Klug, 1974), designated as plus and minus for the fast and slow assembly ends, respectively. Structurally, a microtubule is formed by an arrangement of 13 protofilaments of α- and β-tubulin associated laterally with the β-tubulin pointing towards the plus end and the α-tubulin towards the minus end of the microtubule. There is a cytosolic structure that plays a crucial role in the dynamic microtubules known as the microtubule-organizing center (MTOC), where microtubules are attached at their minus ends and that is involved in the assembly and orientation of microtubules. A third tubulin isoform, γ-tubulin, localizes at MTOCs and is essential for microtubule nucleation, helping the formation of ring structures that serve as templates for microtubule growth (Moritz et al., 1995). During cell division

microtubules localize at the mitotic spindle, thus termed spindle microtubules, and organize and distribute chromosomes to the daughter cells (Karsenti and Vernos, 2001).

Intermediate filaments are made of fibrous proteins that usually adopt a rope-like structure by forming coiled coils, and filament polymerization is independent of nucleotide hydrolysis. There are many different proteins that can form intermediate filaments, which are encoded for by 65 human genes (Oŝhima, 2007). The subunits of the filaments are grouped into five classes: Class 1 and 2 include keratins, class 3 includes vimentin and desmin, class 4 includes neurofilaments (NFs), and class 5 includes nuclear lamins. The expression of these proteins is tissue specific. The intermediate filaments of epithelia are made of keratin subunits while muscle and mesenchymal cell intermediate filaments are made of desmin and vimentin, respectively. On the other hand, NFs are expressed in neurons, and lamins are ubiquitously expressed and localize inside the nuclear envelope of the cells. Despite the structural similarity of different types of intermediate filaments, the amino-acid sequences of the protein subunits are not conserved. However, structural analysis of the protein subunits has allowed some generalizations regarding their functional domains. A conserved central α-helical rod domain is flanked by non-α-helical N-terminal (termed the head) and C-terminal (termed the tail) end domains (Goldman et al., 2008). The central rod enables the formation of a coiled-coil structure, whilst the head and tail domains are variable in size and sequence and contribute to the functional diversity of the intermediate filaments (Steinert and Parry, 1985; Weber and Geisler, 1982). The head and tail domains are important sites of regulation and interaction with other cellular elements. Three modes of intermediate filament polymerization have been described depending on the type of subunit: 1) lamins form

dimers that associate into head-to-tail protofilaments, which further associate laterally (Stuurman et al., 1998); 2) the assembly of vimentin-type filaments starts from the association of two antiparallel vimentin dimers that form a tetramer, followed by lateral annealing and longitudinal elongation, which yield long and thick filaments (Herrmann et al., 1996); and 3) keratin intermediate filaments assemble from heterodimers of acidic and basic keratins, which then associate into tetramers by lateral association and longitudinal elongation (Herrmann et al., 2002).

Intermediate filaments do not contribute to cell motility and no motor proteins are known to be associated with them; thus, it is thought that intermediate filaments play a mainly structural role within the cell. Recently, various skin fragility and muscle disorders, such as Emery-Dreifuss muscular dystrophy, have been shown to be associated with point mutations in genes encoding for intermediate filament proteins (Bonne et al., 1999; Fatkin et al., 1999).

Actin filaments, or microfilaments, are polymers of globular actin (G-actin) (Mommaerts, 1952) that together with a large number of actin binding and associated proteins constitute the actin cytoskeleton. The actin cytoskeleton is dynamic, able to adapt to demands of the cell and is involved in a variety of cellular functions such as locomotion, cell shape regulation, cell division, intracellular trafficking, cell contraction and relaxation, endocytosis, and secretion (Bretscher, 1993; Gottlieb et al., 1993; Juliano and Haskill, 1993; Luna and Hitt, 1992; Salmon, 1989). In addition, the actin cytoskeleton also controls cell-cell and cell-substrate adhesion. Each actin molecule can bind adenosine triphosphate (ATP), which is hydrolyzed to adenosine diphosphate (ADP) after incorporation of an actin molecule into the polymer. Although energy is not required for actin polymerization *in vitro*, ATP-bound actin polymerizes faster than ADP-bound actin (Engel et al., 1977). All subunits in an actin

filament point towards the same end of the filament; thus, an actin filament exhibits polarity.

1.2 Actin

The assembly of G-actin to form F-actin is essential for the actin cytoskeleton to carry out the variety of its cellular functions. Actin is a 43-kDa protein and is present in all eukaryotic cells. There are six genetic isoforms in higher mammals (Vandekerckhove and Weber, 1978): two smooth muscle actins (α-smooth muscle actin and γ-smooth muscle actin), two striated muscle actins (α-skeletal actin and α-cardiac actin) and two cytoplasmic actins (β-cytoplasmic actin and γ-cytoplasmic actin). These actins exhibit a high degree of amino-acid sequence similarity (e.g. skeletal muscle α-actins of human, mouse, rat, rabbit and chicken are identical (Hennessey et al., 1993). Most species have several actin genes, like the worm *Caenorhabdites elegans* that has four distinct actin genes (Krause et al., 1989) and plants that have between five and eight different actins (McLean et al., 1990). On the other hand, lower eukaryotes, like the yeast *Saccharomyces cerevisiae* carry only a single actin gene (Gallwitz and Sures, 1980). The six human actin genes are ACTA1 (α-skeletal actin), ACTA2 (α-smooth muscle actin), ACTB (β-cytoplasmic actin), ACTC (α-cardiac actin), ACTG1 (γ- cytoplasmic actin) and ACTG2 (γ-smooth muscle actin). Mutations in human actin genes have been detected only during the last 10 years due to their low frequencies in the human population and to their severe effects. Mutations in the ACTC gene have been found in several cardiomyopathies (Mogensen et al., 1999; Olson et al., 1998). Mutations in the ACTA1 and ACTG1 genes have been found in congenital myopathies and deafness, respectively (Nowak et al., 1999; Zhu et al., 2003). Interestingly, the majority of actin-related diseases are

dominant due to the fact that most of the mutations produce missense mutations that lead to a defective actin monomer, which is unable to polymerize correctly (Hennessey et al., 1993). Prokaryotic cells have actin homologues, although they share less than 15% sequence homology (Bork et al., 1992). The bacterial actins are termed FtsA, MreB and StbA (or ParM) and their crystal structures show conservation of specific functional motifs that are related to eukaryotic actin, and which are involved in polymerization (Roeben et al., 2006; van den Ent and Lowe, 2000). MreB assembles into filaments that are similar to eukaryotic actin (Jones et al., 2001; van den Ent et al., 2001). The subtle differences in the amino-acid sequences of eukaryotic actin isoforms confer functional diversity and allow actin isoforms to segregate into different structures within cells (Denning et al., 1988; DeNofrio et al., 1989; Otey et al., 1988; Rubenstein et al., 1982).

Under conditions used to grow protein crystals actin monomers have the propensity to spontaneously self-assemble into filaments, hence the first actin monomer crystal structure was determined in the presence of the actin-monomer binding protein DNAse I, which blocks actin polymerization (Kabsch et al., 1990; Mannherz et al., 1977). The crystal structure of actin in the absence of an actin-binding protein has been reported later (Otterbein et al., 2001) (Fig. 1). G-actin displays four prominent domains numbered 1-4. Domains 1 and 2 are separated from 3 and 4 by a cleft that is occupied by an ATP or ADP complexed with a Mg^{2+} ion (Kinosian et al., 1993). The end of the filament at which the ATP/ADP-binding cleft of the actin subunit is exposed is designated the minus or pointed end (domains 2 and 4) while the opposite side is the plus or barbed end (domains 1 and 3).

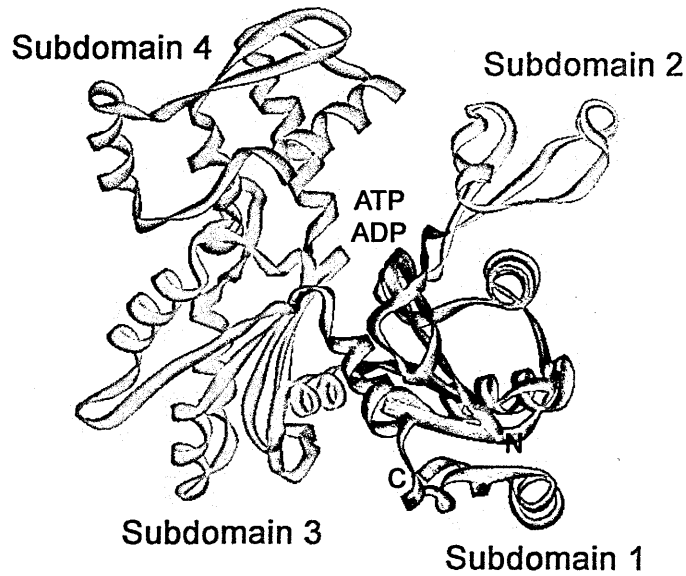

Figure 1. Ribbon representation of the crystal structure of the actin monomer. The four domains are represented in different colors. ADP or ATP is bound at the centre of the molecule, inside the cleft (modified from Otterbein et al., 2001; Kabsch et al., 1990).

1.3 Assembly and regulation of actin filaments

Unlike the actin monomer, no atomic structure has been solved for F-actin, although models have been proposed (Holmes et al., 1990) and low level structural data have been presented (Oda et al., 2001; Popp et al., 1987) (Fig.2). Each monomer is found surrounded by four others in a filament. Actin filaments are constantly polymerizing and depolymerizing inside the cell. Association and dissociation of actin monomers from the filament can occur at both ends, but association predominantly occurs at the barbed end and dissociation from the pointed end (Wegner, 1976).

In vitro under low ionic strength conditions, e.g., 0.2 mM Ca^{2+} or 0.05 mM Mg^{2+}, actin is monomeric, and elevating the ionic strength to physiological conditions, e.g., 2 mM $MgCl_2$ or 100 mM KCl, causes monomeric G-actin to polymerize into synthetic F-actin (Kasai et al., 1962b). *In-vitro* polymerization of actin occurs in three steps (Kasai et al., 1962a; Kasai et al., 1962b). The first is the nucleation phase, which is characterized by an energetically unfavourable process of aggregating actin monomers to form a stable trimer, which can support the addition of further actin monomers for the formation of actin filaments. The elongation phase is the second step and filaments are rapidly formed in this phase. At the third step, a steady-state phase is reached, and there is an equilibrium between filaments and monomers. Exchange of actin monomers continues at the ends of the filament, but the total mass remains the same. The flux of actin subunits between the pointed end and the barbed end is known as treadmilling (Wegner, 1976). The rate of treadmilling or the assembly/disassembly process of actin filaments is regulated by actin-binding proteins (ABPs). Some of the functions of ABPs include severing or capping of actin filaments, nucleating actin monomers for polymerization, and filament cross-linking

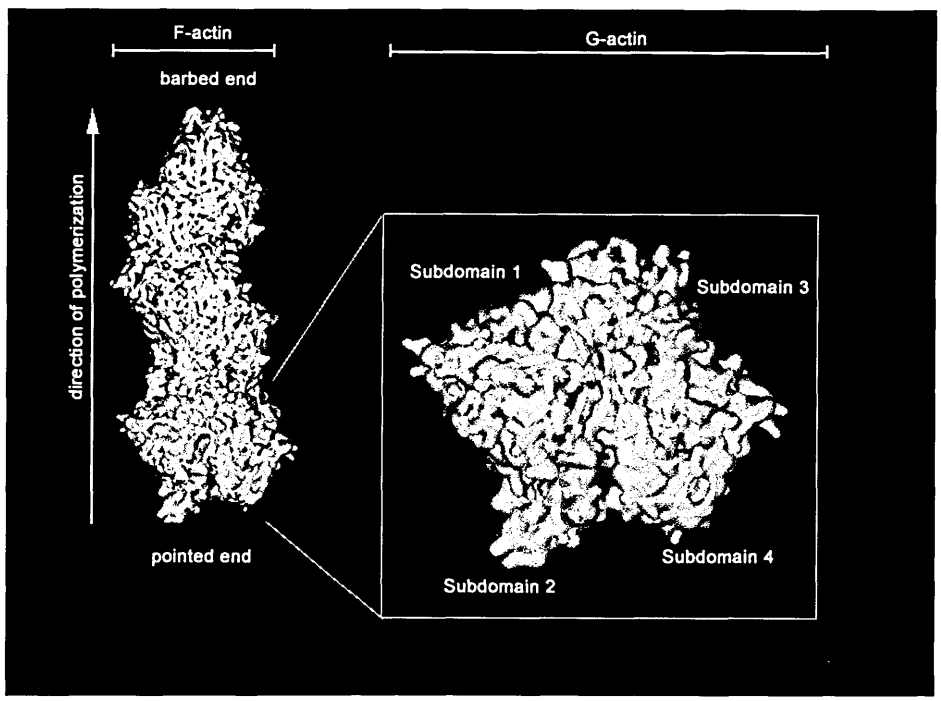

Figure 2. Model of the F-actin structure. The F-actin model comprises five actin monomers (blue, red, green, white and yellow). Each actin monomer is surrounded by four others in a filament (green monomer). Association of monomers occurs at the barbed end, and dissociation from the pointed end. During actin filament maturation, ATP-G-actin is converted to ADP-G-actin.

and stabilization. Other ABPs can bind and sequester actin monomers. Several toxins can promote depolymerization or stabilization of actin filaments. Cytochalasin is a fungal alkaloid that binds to the plus ends of the actin filaments, thus inhibiting the association and dissociation of actin subunits at that end, and resulting in the depolymerization of the actin filament (Cooper, 1987). Latrunculin is another toxin found in the sponge *Latrunculia magnifica* (Spector et al., 1983) and it binds and sequesters actin monomers and prevents their addition to actin filaments, thus also resulting in the depolymerization of the filaments (Coue et al., 1987). Phalloidin is a bicyclic heptapeptide purified from the poisonous mushroom *Amanita* (Lengsfeld et al., 1974). In contrast to latrunculin and cytochalasin, phalloidin binds to the actin filament, thus stabilizing it and protecting it from depolymerization. These three toxins are widely used for actin cytoskeleton studies due to their specificities.

The functional diversity of ABPs dictates (or defines) the multiple and distinct roles of actin filaments in the cell. Filaments at the leading edge of a migrating cell (lamellipodium) are dynamic and compositionally different from actin filaments that are responsible for contraction in skeletal, cardiac and smooth muscle cells and also from the actin filaments of the microvilli that are found at the apical surface in epithelial cells of the intestine.

1.3.1 Actin filaments in motile cells

The actin cytoskeleton plays an important role in eukaryotic cell motility, and many processes depend on locomotion including embryonic development, chemotactic movement of immune cells, and fibroblast migration. Movement is characterized by cytoplasmic membrane extensions termed pseudopods and depending on their morphology, are called lamellipodia (observed at the leading edge

of many migrating cells; Fig. 3A), filopodia (spike-like extensions) or ruffles (similar to lamellipodia but with more membrane irregularities; Fig. 3B). Behind the lamellipodia lie bundles of actin filaments termed stress fibres, which are held together by actin-crosslinking proteins (e.g. α-actinin and filamin). Following the classification of Small, stress fibres can be divided into three classes on the basis of their subcellular location (Small et al., 1998): 1) ventral stress fibres, 2) dorsal stress fibres and 3) transverse arcs (Fig. 3C and D). Ventral stress fibres are positioned along the base of the cell, attached at both ends to focal adhesions, which are structures that connect the actin cytoskeleton with the extracellular matrix. Dorsal stress fibres are attached to focal adhesions from one end at the base of the cell and ascend towards the dorsal surface (Heath and Dunn, 1978). Transverse arcs are actin bundles that form beneath the dorsal surface and behind the lamellipodia (Heath, 1983).

Each type of stress fibre is assembled by a different mechanism and associated with different ABPs. Dorsal stress fibres elongate from focal adhesions. Ventral stress fibres are formed by end-to-end joining of two dorsal stress fibres rendering a structure that is anchored at both ends by focal adhesions (Fig. 4). In contrast, transverse arcs form by joining small actin bundles and they are not attached to focal adhesions (Hotulainen and Lappalainen, 2006). An important component of stress fibres is the motor protein myosin, which is found associated with actin and forms an actomyosin complex. Myosin is essential for stress fibre contraction, although polarity alternation of the actin filaments within a stress fibre is also important (Cramer et al., 1997). Polarity alternation of actin filaments allows their movement in opposite directions by myosin, which results in contraction. Dorsal stress fibres show uniform polarity, thus it is thought that they do not contract. On the other hand, ventral and

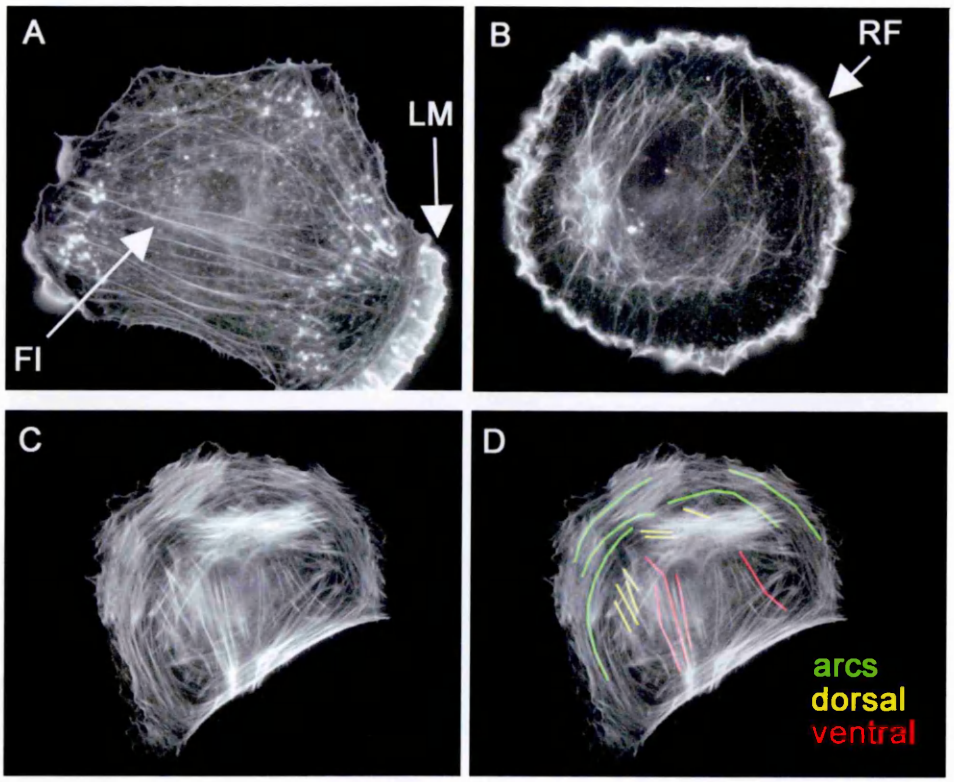

Figure 3. A7r5 smooth muscle cells stained with phalloidin. (A) Cell showing a polarized lamellipodia and long ventral stress fibres. (B) Ruffles are seen at the periphery of the cell. (C) and (D) shows the three classes of filaments following the classification of Small; ventral stress fibres (red), dorsal stress fibres (yellow) and transverse arcs (green). [Fl] actin filament, [LM] lamellipodia, [RF] membrane ruffles.

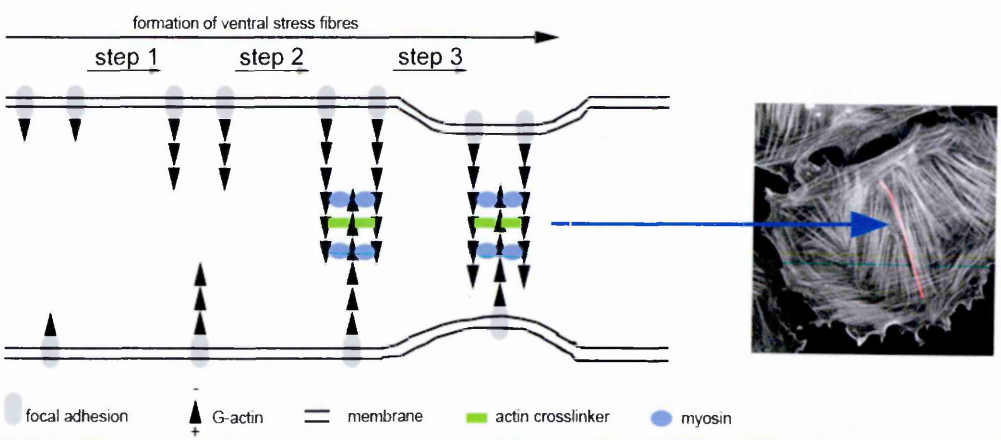

Figure 4. Formation of a ventral stress fibre. Actin filaments polymerize from focal adhesions towards the interior of the cell (step 1). Actin crosslinking proteins (green) crosslink filaments and can stabilize them in an antiparallel fashion (step 2). The antiparallel organization of actin filaments is exploited by myosin (blue), to move the filaments and generate a contractile force that is transmitted to the actin network (step 3).

transversal arc stress fibres show polarity alternation since they are made by end-to-end joining of shorter filaments.

1.3.2 Regulation of actin filaments at the leading edge of motile cells

The extension of lamellipodia in migrating cells is an actin-based process that is highly polarized, and specific molecular components are required for the dynamic assembly of this cytoskeletal network. Interestingly, actin fibres of the lamellipodia arrange in uniform parallel arrays similar to dorsal stress fibres. Protrusion at the leading edge is regulated by the actin-related protein (Arp) 2/3 complex. Arp2/3 is a multimeric protein complex that regulates the actin cytoskeleton in many different ways (Goley and Welch, 2006). The Arp2/3 complex binds to actin filaments, creates 70° branches and acts as a crosslinker (Fig. 5). The Arp2/3 complex consists of seven polypeptides termed Arp2 of 45-kDa, Arp3 of 47-kDa, actin-related protein complex (Arpc) 1 of 41-kDa, Arpc2 of 34-kDa, Arpc3 of 21-kDa, Arpc4 of 20-kDa and Arpc5 of 16-kDa (Goley and Welch, 2006). Arp2/3 promotes actin filament formation by the generation of templates that nucleate actin polymerization (Welch et al., 1997) and initiates actin filament branches by binding to the sides of existing filaments (Fujiwara et al., 2002; Mullins et al., 1998; Volkmann et al., 2001). The result of this process is an active polymerization process of actin filaments that pushes the membrane forward.

Three different ways used by cells to trigger actin polymerization have been proposed (Higgs and Pollard, 1999): 1) *de novo* nucleation of filaments from monomeric actin; 2) severing of existing filaments to create uncapped barbed ends and; 3) uncapping of existing barbed ends. Until the discovery of the Arp2/3 complex, no protein was known to have an actin-nucleating activity. The nucleation activity of

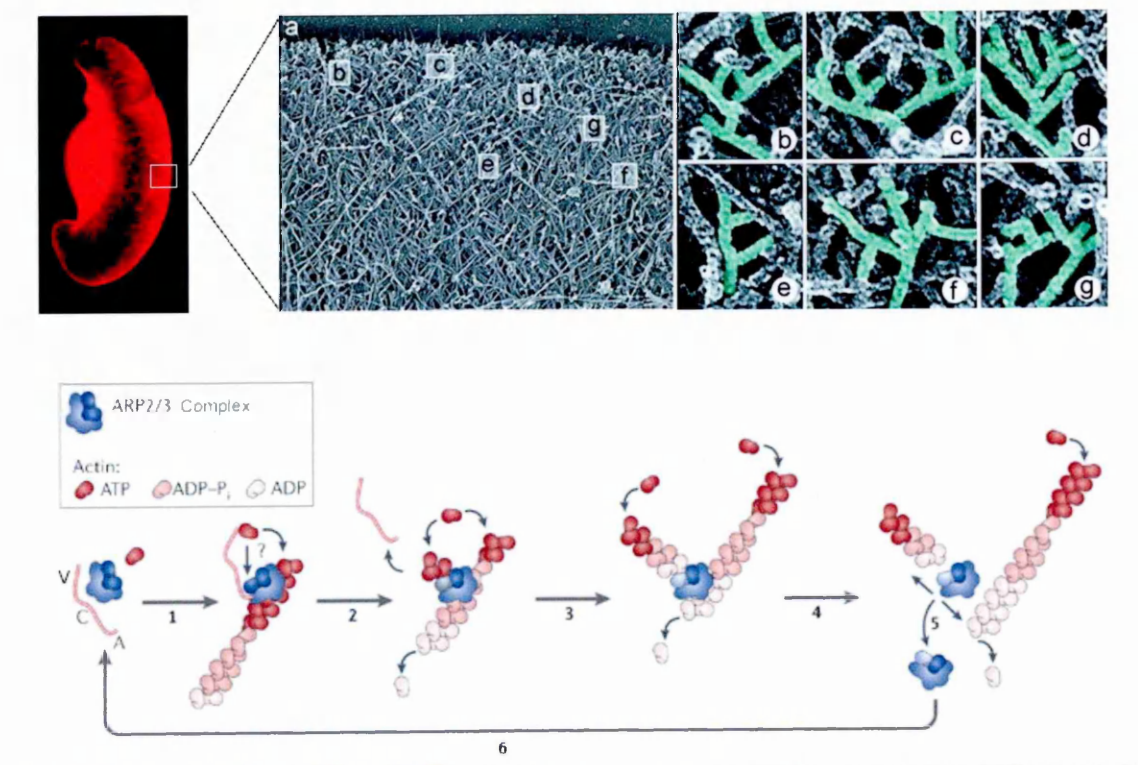

Figure 5. Upper panel: Multiple branching of actin filaments in lamellipodia. electron micrograph of lamellipodia of Xenopus keratocytes (a-g) showing an overview of the leading edge (a) and enlargements of the boxed regions (b-g). Many examples of filaments with multiple branches (cyan) can be visualized in lamellipodia despite the high overall density of the actin network (from Svitkina and Borisy, 1999).

Lower panel: Scheme of nucleation of actin polymerization by the Arp2/3 complex based on Goley and Welch, 2006. The Arp2/3 complex is shown in blue and different nucleotide state of actin is indicated in red shadings. The cycle starts with the activation of Arp2/3 by the VCA domains of WASp, which promotes association to the actin filament (Step 1). ATP-G-actin is nucleated and a daughter filament elongates (steps 2 and 3). Filament aging weakens the interaction between Arp2/3 and actin filaments, allowing branch disassembly (steps 4 and 5). Arp2/3 is released and recycled (step 6).

Arp2/3 is directly regulated by members of the Wiskott-Aldrich syndrome (WASp)/Scar protein family (Machesky et al., 1999).

The WASp/Scar family includes WASp of 53-kDa, neuronal WASp (N-WASp) of 55-kDa, and the 62-kDa WASP family Verprolin-homologueous protein (WAVE, which is the vertebrate homologue of Scar). This protein family interacts with a variety of molecules known to influence cytoskeletal dynamics, and is considered to integrate diverse cellular signals (e.g. growth factor receptor signals) through their functional domains with the actin cytoskeleton. The WASp/Scar proteins share similar C-terminal WASp homology 2 (WH2), VCA (Verproline homology, Cofilin homology, Acidic) domains and proline-rich central regions. However, differences are seen in the N-terminus (e.g. N-WASp carries an IQ domain and WAVE lacks a WH1 domain found in WASp and N-WASp) (Zigmond, 2000). The VCA domain is sufficient to bind the Arp2/3 complex and to activate its nucleation activity (Machesky et al., 1999; Rohatgi et al., 1999). It has been shown for N-WASp that the N-terminal WH1 domain interacts with the C-terminal VCA domain and inhibits the activation of Arp2/3. In addition, the Rho family GTPase Cdc42 can bind to the WH1 domain and block the intramolecular interaction between the C- and N- termini, resulting in activation of Arp2/3 (Rohatgi et al., 2000). However, whether this regulatory mechanism also occurs in the other members of the WASp/Scar family is not known. The proline-rich region binds multiple src-homology 3 (SH3) domain-containing proteins (Bunnell et al., 1996; Finan et al., 1996). N-WASp and WASp carry a GTPase-binding domain (GBD), which confers the ability to interact with activated Cdc42 and Rac (Symons et al., 1996). Cdc42 and Rac belong to the Rho family of GTPases, which are known to influence different aspects of the actin cytoskeleton (e.g. formation of stress fibres, filopodia and ruffles). On the other hand,

WAVE lacks a GBD, although it was shown to be regulated by Rac (Miki et al., 1998b). Another difference between WAVE, WASp and N-WASp is the presence of a WH1 domain in the latter two proteins, which can bind phosphatidylinositol (4,5)-bisphosphate (PIP_2). PIP_2 activates WAVE and WASp, hence activating Arp2/3 nucleation activity (Miki et al., 1996).

1.3.3 Severing, capping and uncapping actin filaments

Actin polymerization can occur *in vivo* by nucleation of unpolymerized actin (e.g. mediated by Arp2/3) or by the creation of free barbed ends by actin severing proteins, or by the release of capping proteins from the tip of the filament. Gelsolin, and capping protein (CapZ) are two well studied proteins that cap barbed ends of actin filaments (Bryan and Coluccio, 1985; Caldwell et al., 1989) and are both released upon polyphosphoinositide binding (e.g. PIP_2) (Janmey and Stossel, 1987; Schafer et al., 1996),

CapZ is a heterodimeric protein that is composed of an α-subunit of 33-kDa and a β-subunit of 31-kDa. Association of CapZ with actin filaments prevents the exchange of actin subunits from the barbed end. CapZ also attaches actin filaments to other structures such as cell-cell junctions and the Z-line of skeletal and cardiac muscle, and CapZ help the formation of actin nuclei for the elongation of filaments (Casella et al., 1987; Casella et al., 1986; Casella and Torres, 1994).

Gelsolin is an 80-kDa protein able to break and cap actin filaments, thus producing short filaments that cannot elongate (Sun et al., 1999). The release of intracellular Ca^+ upon an external stimulus activates gelsolin to sever actin filaments (Yin et al., 1980) and cap their barbed ends. In addition, membrane-bound PIP_2 can remove the gelsolin cap and contribute to actin polymerization (Hartwig et al., 1995).

One gelsolin isoform is released into the blood stream, where it severs and caps actin filaments released into the circulation, in order to be ultimately removed from the circulation by the liver (Lind et al., 1986). However, severing and (un)capping processes alone are not sufficient to drive actin polymerization. Pools of actin monomers have to be permanently available in order to be incorporated at the barbed ends.

1.3.4 Actin-monomer-binding proteins

Non-muscle cells maintain a high concentration of unpolymerized actin (≤100 μM) and the vast majority is bound to ATP. Interestingly, 100 μM of actin monomers in physiological concentrations of salt polymerize rapidly *in vitro*, leaving only 0.1 μM of monomers (known as the critical concentration). Hence, a regulatory mechanism must exist in cells to maintain the high levels of actin monomers (Pollard et al., 2000). Indeed, actin-monomer-binding proteins regulate actin polymerization and depolymerization by sequestering G-actin, exchanging the nucleotide ADP for ATP and delivering the monomer to the barbed end of the filament.

The best-characterized actin-monomer-binding proteins are the actin depolymerization factor (ADF) /cofilin, twinfilin, WASp/Scar (see Sec. 1.3.2), thymosin and profilin. ADFs and cofilins, both of 18.5-kDa are able to increase actin filament turnover by binding ADP-actin monomers and increasing the dissociation rate of actin from the pointed ends of the filaments, but not from the barbed ends (Carlier et al., 1997). In addition, ADF/cofilins can sever (Nishida et al., 1984) and bind actin filaments (Hayden et al., 1993). ADF and cofilins can bind ADP-actin monomers with greater affinity than ATP-actin, and have greater affinity for ADP-actin filaments than ATP-actin filaments or ADP+Pi-actin filaments (Maciver and

Weeds, 1994). ADF/cofilins are also present at the contractile ring during cytokinesis and are necessary for its depolymerization (Gunsalus et al., 1995; Nagaoka et al., 1995), and the *Drosophila* cofilin homologue has been shown to be essential for cell division (Gunsalus et al., 1995).

Twinfilin is a 44-kDa protein that sequesters actin monomers in a 1:1 manner and binds ADP-actin preferentially, inhibiting the exchange of ADP for ATP (Goode et al., 1998) and thus affecting actin polymerization. In addition, twinfilin possesses two ADF/cofilin-like domains, which are ~20% homologueous to ADF/cofilin and are important for G-actin binding (Lappalainen et al., 1998; Palmgren et al., 2001). The actin-monomer-binding activity can be regulated by PIP_2, and binding of PIP_2 *in vitro* downregulates the actin-monomer-sequestering activity of twinfilin.

Thymosins are small proteins of 5-kDa that sequester G-actin through their WH2 domain (Paunola et al., 2002) and prevent the delivery of actin monomers to the barbed end of the filaments. Profilins, with an approximate molecular weight of 19-kDa, also sequester G-actin, but unlike thymosins they can bind the barbed end of the actin filament and promote filament polymerization (Tilney et al., 1983).

Actin-monomer-binding proteins can distinguish between ATP-actin and ADP-actin (e.g. thymosin binds about 50-fold more strongly to ATP-actin than to ADP-actin) (Carlier et al., 1993) and they can also compete for the same actin-binding site (e.g. thymosin-β4 competes with profilin for binding to overlapping sites on actin) (Pantaloni and Carlier, 1993; Schutt et al., 1993). It has been shown that actin bound to thymosin does not polymerize, but that profilin can displace thymosin from actin monomers and transport actin to the barbed end of actin filaments. A similar mechanism is observed between ADF/cofilin and twinfilin, which share similar actin-binding sites. Depolymerized ADP-actin monomers could be delivered to twinfilin by

ADF/cofilin, and twinfilin may keep the actin monomers in their ADP form near the barbed ends (Goode et al., 1998). Twinfilin could release the ADP-actin monomer at rapid polymerization sites where the actin monomer would undergo nucleotide exchange and assemble into the plus end of the filament either spontaneously or mediated by profilin (Palmgren et al., 2002). Thus, actin-monomer-binding proteins can be classified into monomer-sequestering proteins (thymosin and twinfilin), ATP-monomer-binding proteins (profilin and thymosin), ADP-monomer-binding proteins (ADF/cofilin, twinfilin), depolymerization promoters (ADF/cofilin), and actin-ADP nucleotide exchange inhibitors (twinfilin and cofilin). The correct spatial and temporal organization of actin filaments and of their regulatory proteins is essential for the correct function of eukaryotic cells. In contrast to the dynamic pool of actin filaments at the cell cortex or in lamellipodia, a less dynamic and more stable pool of actin filaments is distinguished in many cultured cells and tissues. These filaments can be found packed into stress fibres and distribute through the cytoplasm.

1.3.5 Properties of stable actin filaments

Highly dynamic actin filaments and low dynamic actin-rich structures can coexist in migrating cells (e.g. stress fibres behind the leading edge of migrating cells), and many cells form specialized actin-based structures that are relatively stable (e.g. myofibrils of the striated muscle and the long bundles of actin filaments in microvilli of the intestinal brush border). The half-life of fluorescence recovery after photobleaching (FRAP) for rhodamine-labelled muscle actin in striated muscles is ~60 min (Shimada et al., 1997), which is relatively slow compared to other actin structures, such as stress fibres (~5-10 min) (Kreis et al., 1979) or lamellipodia in migrating cells (~0.5-3.0 min) (Theriot and Mitchison, 1991). A variety of ABPs are

able to stabilize actin filaments (e.g. calponin), anchor the actin cytoskeleton the cell membrane (e.g. α-actinin and spectrin), affect the elasticity of the actin network (e.g. spectrin), crosslink actin filaments (e.g. fimbrin, fascin and α-actinin) or displace actin filaments (e.g. myosin).

Formation of stable actin filaments into higher order structures is controlled by proteins that can crosslink and bundle actin filaments (e.g. α-actinin, spectrin and fimbrin), and many of these proteins share a conserved domain that mediates their association with actin filaments (Puius et al., 1998). This actin-binding domain (ABD) consists of a tandem repeat of two calponin-homology (CH) domains (Gimona et al., 2002). Crosslinking and bundling proteins utilize two ABDs to connect actin filaments. Fimbrin is a 70-kDa protein that carries two ABDs arranged in tandem on the same polypeptide chain (Goldsmith et al., 1997), which allows the formation of tightly packed bundles of actin filaments (Matsudaira, 1994). In contrast, many other crosslinking proteins, such as α-actinin, carry a single ABD; however, these proteins form dimers, thus exposing two ABDs, which are necessary to crosslink or bundle actin filaments (Broderick and Winder, 2002). It is known that the distance between two ABDs of an actin crosslinking protein correlates with the structural properties of the actin filament network (Tseng et al., 2005). The ABDs of filamin are separated by a flexible arm and crosslink actin filaments mostly into an orthogonal network (Tseng et al., 2004), while α-actinin ABDs are separated by a 30-40 nm spacer, and crosslink and bundle actin filaments into tight, parallel arrays (Meyer and Aebi, 1990; Wachsstock et al., 1993). In addition, the formation of different types of actin filament arrays are concentration dependent. Low concentrations of α-actinin induce formation of loosely packed actin filaments, whereas high concentrations induce tightly packed parallel arrays of actin filaments.

α-Actinin belongs to the spectrin superfamily of proteins, which includes dystrophin, utrophin and spectrin (Baron et al., 1987; Davison and Critchley, 1988; Koenig et al., 1988). Members of the spectrin superfamily have an ABD at their N-terminus that is followed by multiple spectrin repeats, which are responsible for protein dimerization. These repeats vary in length among the members of the superfamily, and they determine the flexibility of the ABP and the nature of the actin filament network; at the C-terminus a calcium-binding EF-hand domain is commonly present (Broderick and Winder, 2002). While α-actinin forms antiparallel homodimers, spectrin is found as heterotetramers of α- and β-spectrin; no dimerization has been found for dystrophin (Chan and Kunkel, 1997; Davison et al., 1989; Kahana and Gratzer, 1991). Each α-actinin subunit has a molecular weight of ~103-kDa, and four genetic isoforms have been described, each found within specific tissues. α-Actinin-1 and 4 are mainly found in non-muscle cells and are sensitive to regulation by Ca^{2+} (Burridge and Feramisco, 1981). They are both associated with focal adhesions and stress fibres, however, in motile cells, α-actinin-4 is also found in ruffles (Araki et al., 2000; Edlund et al., 2001; Pavalko and Burridge, 1991). In contrast, α-actinin-2 and 3 are found in skeletal, cardiac and smooth muscle cells and are insensitive to Ca^{2+} (Beggs et al., 1992).

Spectrin is an antiparallel heterodimer that is composed of β- and α-spectrin with molecular weights of 246-kDa and 280-kDa, respectively, and it has been extensively characterized in erythrocytes (Stokke et al., 1986), where the flexibility of spectrin contributes to the inherent deformability of red blood cells (Elgsaeter et al., 1986). Mutations in spectrin are associated with hereditary haemolytic anaemia (Delaunay and Dhermy, 1993; Wichterle et al., 1996) where red blood cell shape is abnormal and the cells are sensitive to haemolysis. In contrast to α-actinin, spectrin

also possesses a pleckstrin-homology (PH) and SH3 domains, which allow binding to membrane lipids and interactions with proline-rich regions, respectively (Chakrabarti et al., 2006). Although the actin-binding activity of spectrins from erythrocytes is insensitive to Ca^{2+} regulation, the ability to bind actin filaments in other cells is modulated by Ca^{2+} (Dubreuil et al., 1991; Fishkind et al., 1987; Wallis et al., 1992).

Dystrophin is a 427-kDa protein (Dp427) (Koenig et al., 1988), and it carries an ABD at its N-terminus, followed by 24 spectrin-like repeats. Mutations in the dystrophin gene are associated with many muscular diseases such as Duchenne and Becker muscular dystrophy where the regenerative capacity of the muscle is lost. There are several alternatively spliced isoforms of dystrophin such as Dp260 (D'Souza et al., 1995), Dp140 (Lidov et al., 1995), Dp116 (Byers et al., 1993) and Dp71 (Blake et al., 1992). Dystrophin isoforms are specifically expressed in different tissues. Unlike the other members of the spectrin family, dystrophin does not bundle or crosslink actin filaments (Rybakova et al., 1996). The C-terminus contains a WW domain, which binds to proline-rich substrates and allows interactions with other proteins. Utrophin is a protein of 395-kDa, and it is a genetic isoform of dystrophin with major differences in the C-terminus (Love et al., 1989).

Besides the well-characterized spectrin protein family other crosslinking and bundling proteins have been described that posses an ABD formed by two CH domains. These proteins include filamin, plectin, cortexillin, smoothelin, fimbrin and fascin, which are reviewed briefly below.

Filamin is a parallel homodimer and each subunit has a molecular weight of 280-kDa. Filamin subunits consist of an N-terminal ABD followed by a rod-shape domain, which is important for dimerization (Weihing, 1988). There are three genetic isoforms, FLNA, FLNB and FLNC. Many human diseases have been related to

mutations in FLNA and FLNB, which cause abnormal development of brain, bone, the cardiovascular system and many other organs (Eksioglu et al., 1996; Kakita et al., 2002; Robertson et al., 2003). Dimerization of filamins through the last C-terminal repeat allows the formation of a V-shaped flexible structure (Gorlin et al., 1990). The flexibility of filamins allows it to crosslink actin filaments orthogonally (Weihing, 1985).

Plectin is a functionally versatile protein, which binds to several components of the cytoskeleton such as actin, intermediate filaments and microtubules (Foisner et al., 1991; Wiche et al., 1982; Yang et al., 1996). The molecular weights of plectin isoforms vary from 507-kDa to 527-kDa and they carry an ABD at their N-termini, which allows binding to actin filaments.

Cortexillins also possess an ABD at their N-termini, but unlike the spectrins, filamins and plectins, they also posses a second strong actin-binding site (ABS) at their C-termini (Stock et al., 1999) which is not related to CH domains. Cortexillins are made of parallel heterodimers of cortexillin I and II isoforms (Faix et al., 1996) with an approximate molecular weights of 50-kDa each.

Smoothelins share similar functions with the other actin crosslinkers and bundlers, however a major difference is observed in its single CH domain, which is positioned at the C-terminus of the protein (Gimona et al., 2002; Niessen et al., 2004). Two spliced isoforms have been described so far with molecular weights of 59-kDa (smoothelin A) and 110-kDa (smoothelin B). Smoothelins that lack the CH domain still bind actin filaments, and thus it is thought that more than one ABS exists on the molecule.

Fascins are 55-58-kDa proteins that form tight and stable bundles of actin filaments (Otto et al., 1979; Saishin et al., 1997). Three genetic isoforms are found in

humans, fascin-1, -2 and –3. All of these contain an ABD at their C-terminus and a second unrelated ABS at their N-terminus. Fascin-1 is expressed in most tissues, whilst fascin-2 is expressed in the retina, and fascin-3 expression is largely restricted to the testis (Kureishy et al., 2002). Fascins colocalize with filopodia and it is believed that they are key elements of filopodia formation. The inclusion of fascins with actin in liposomes causes the extension of straight and rigid projections from their surface (Honda et al., 1999).

1.4 Signalling to the actin cytoskeleton

Cells have evolved complex mechanisms to sense and respond to the external environment. Whether keratocytes migrate towards wound areas or neutrophils cross endothelial cells to escape from the blood stream, both need massive re-arrangements of the actin cytoskeleton to perform these tasks (Radice, 1980; Zigmond and Lauffenburger, 1986). Hence, cells can adapt the actin cytoskeleton according to their needs. External signals, such as growth factors, pro-inflammatory molecules or components of the extracellular matrix can bind to membrane receptors that can then lead to the activations of specific signalling pathways. Signalling pathways involve enzymes that catalyze reactions such as (de)phosphorylation and nucleotide exchange. It is possible to distinguish between an input and an output control. The input control determines the temporal and spatial activation of the enzymes. The output control determines the spatial and temporal activation of their downstream partners.

ABPs, which are the major actin-regulatory proteins, are targeted by many cell-signalling proteins and molecules (e.g. PIP_2, Ca^{2+} and PKC) that can activate or inactive them. The activation of many signalling pathways results in Ca^{2+} release and the generation of different types of phosphatidylinositols (PIs). Both tightly regulate

the assembly of actin polymers. Ca^{2+} and PIs can control the activity of capping, monomer-binding and severing proteins. The capping and monomer-binding proteins profilin and CapZ contain a PIP_2 binding site and binding of the phosphoinositide inhibits their association with actin (Heiss and Cooper, 1991). On the other hand, the severing activity of gelsolin can be regulated either by Ca^{2+} or by PIP_2 (Bearer, 1991; Janmey et al., 1987; Yin and Janmey, 2003). Binding of Ca^{2+} to gelsolin activates its severing activity, in contrast to PIP_2, which inhibits gelsolin activity. Also ADF and cofilin activities are inhibited by phosphatidylinositols (Yonezawa et al., 1990). Ca^{2+} and PIs also regulate actin crosslinking proteins. α-Actinin crosslinking activity is enhanced by PIP and PIP_2, and α-actinin-1 and -4 activity is dependent on Ca^{2+} (Fukami et al., 1992; Noegel et al., 1987; Witke et al., 1993). In contrast, filamin actin-crosslinking activity is reduced by PIP and PIP_2 (Furuhashi et al., 1992).

1.4.1 Organization of the actin cytoskeleton by Rho GTPases

The Rho GTPase protein family is known to be a key regulator of signalling pathways that link extracellular and intracellular stimuli to the organization of the cytoskeleton. Signalling through Rho GTPases can be initiated by activation of many different types of plasma membrane receptors (Hall and Nobes, 2000). Rho GTPases are key players in the formation of lamellipodia, membrane ruffles, filopodia and of stable stress fibres. Rho GTPases cycle between an active GTP-bound form and an inactive GDP-bound form. The GTP-bound active form of RhoGTPases activates a plethora of downstream effectors. The cycle between the active and inactive state is regulated by GDP/GTP exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs; Fig. 6). GEFs are responsible for the conversion of the inactive GDP-bound form of the GTPases to the active GTP-

Arp2/3 is directly regulated by members of the Wiskott-Aldrich syndrome (WASp)/Scar protein family (Machesky et al., 1999).

The WASp/Scar family includes WASp of 53-kDa, neuronal WASp (N-WASp) of 55-kDa, and the 62-kDa WASP family Verprolin-homologueous protein (WAVE, which is the vertebrate homologue of Scar). This protein family interacts with a variety of molecules known to influence cytoskeletal dynamics, and is considered to integrate diverse cellular signals (e.g. growth factor receptor signals) through their functional domains with the actin cytoskeleton. The WASp/Scar proteins share similar C-terminal WASp homology 2 (WH2), VCA (Verproline homology, Cofilin homology, Acidic) domains and proline-rich central regions. However, differences are seen in the N-terminus (e.g. N-WASp carries an IQ domain and WAVE lacks a WH1 domain found in WASp and N-WASp) (Zigmond, 2000). The VCA domain is sufficient to bind the Arp2/3 complex and to activate its nucleation activity (Machesky et al., 1999; Rohatgi et al., 1999). It has been shown for N-WASp that the N-terminal WH1 domain interacts with the C-terminal VCA domain and inhibits the activation of Arp2/3. In addition, the Rho family GTPase Cdc42 can bind to the WH1 domain and block the intramolecular interaction between the C- and N- termini, resulting in activation of Arp2/3 (Rohatgi et al., 2000). However, whether this regulatory mechanism also occurs in the other members of the WASp/Scar family is not known. The proline-rich region binds multiple src-homology 3 (SH3) domain-containing proteins (Bunnell et al., 1996; Finan et al., 1996). N-WASp and WASp carry a GTPase-binding domain (GBD), which confers the ability to interact with activated Cdc42 and Rac (Symons et al., 1996). Cdc42 and Rac belong to the Rho family of GTPases, which are known to influence different aspects of the actin cytoskeleton (e.g. formation of stress fibres, filopodia and ruffles).

On the other hand, WAVE lacks a GBD, although it was shown to be regulated by Rac (Miki et al., 1998b). Another difference between WAVE, WASp and N-WASp is the presence of a WH1 domain in the latter two proteins, which can bind phosphatidylinositol (4,5)-bisphosphate (PIP_2). PIP_2 activates WAVE and WASp, hence activating Arp2/3 nucleation activity (Miki et al., 1996).

1.3.3 Severing, capping and uncapping actin filaments

Actin polymerization can occur *in vivo* by nucleation of unpolymerized actin (e.g. mediated by Arp2/3) or by the creation of free barbed ends by actin severing proteins, or by the release of capping proteins from the tip of the filament. Gelsolin, and capping protein (CapZ) are two well studied proteins that cap barbed ends of actin filaments (Bryan and Coluccio, 1985; Caldwell et al., 1989) and are both released upon polyphosphoinositide binding (e.g. PIP_2) (Janmey and Stossel, 1987; Schafer et al., 1996),

CapZ is a heterodimeric protein that is composed of an α-subunit of 33-kDa and a β-subunit of 31-kDa. Association of CapZ with actin filaments prevents the exchange of actin subunits from the barbed end. CapZ also attaches actin filaments to other structures such as cell-cell junctions and the Z-line of skeletal and cardiac muscle, and CapZ help the formation of actin nuclei for the elongation of filaments (Casella et al., 1987; Casella et al., 1986; Casella and Torres, 1994).

Gelsolin is an 80-kDa protein able to break and cap actin filaments, thus producing short filaments that cannot elongate (Sun et al., 1999). The release of intracellular Ca^+ upon an external stimulus activates gelsolin to sever actin filaments (Yin et al., 1980) and cap their barbed ends. In addition, membrane-bound PIP_2 can remove the gelsolin cap and contribute to actin polymerization (Hartwig et al., 1995).

One gelsolin isoform is released into the blood stream, where it severs and caps actin filaments released into the circulation, in order to be ultimately removed from the circulation by the liver (Lind et al., 1986). However, severing and (un)capping processes alone are not sufficient to drive actin polymerization. Pools of actin monomers have to be permanently available in order to be incorporated at the barbed ends.

1.3.4 Actin-monomer-binding proteins

Non-muscle cells maintain a high concentration of unpolymerized actin (≤100 μM) and the vast majority is bound to ATP. Interestingly, 100 μM of actin monomers in physiological concentrations of salt polymerize rapidly *in vitro*, leaving only 0.1 μM of monomers (known as the critical concentration). Hence, a regulatory mechanism must exist in cells to maintain the high levels of actin monomers (Pollard et al., 2000). Indeed, actin-monomer-binding proteins regulate actin polymerization and depolymerization by sequestering G-actin, exchanging the nucleotide ADP for ATP and delivering the monomer to the barbed end of the filament.

The best-characterized actin-monomer-binding proteins are the actin depolymerization factor (ADF) /cofilin, twinfilin, WASp/Scar (see Sec. 1.3.2), thymosin and profilin. ADFs and cofilins, both of 18.5-kDa are able to increase actin filament turnover by binding ADP-actin monomers and increasing the dissociation rate of actin from the pointed ends of the filaments, but not from the barbed ends (Carlier et al., 1997). In addition, ADF/cofilins can sever (Nishida et al., 1984) and bind actin filaments (Hayden et al., 1993). ADF and cofilins can bind ADP-actin monomers with greater affinity than ATP-actin, and have greater affinity for ADP-actin filaments than ATP-actin filaments or ADP+Pi-actin filaments (Maciver and

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Plectin is a functionally versatile protein, which binds to several components of the cytoskeleton such as actin, intermediate filaments and microtubules (Foisner et al., 1991; Wiche et al., 1982; Yang et al., 1996). The molecular weights of plectin isoforms vary from 507-kDa to 527-kDa and they carry an ABD at their N-termini, which allows binding to actin filaments.

Cortexillins also possess an ABD at their N-termini, but unlike the spectrins, filamins and plectins, they also posses a second strong actin-binding site (ABS) at their C-termini (Stock et al., 1999) which is not related to CH domains. Cortexillins are made of parallel heterodimers of cortexillin I and II isoforms (Faix et al., 1996) with an approximate molecular weights of 50-kDa each.

Smoothelins share similar functions with the other actin crosslinkers and bundlers, however a major difference is observed in its single CH domain, which is positioned at the C-terminus of the protein (Gimona et al., 2002; Niessen et al., 2004). Two spliced isoforms have been described so far with molecular weights of 59-kDa (smoothelin A) and 110-kDa (smoothelin B). Smoothelins that lack the CH domain still bind actin filaments, and thus it is thought that more than one ABS exists on the molecule.

Fascins are 55-58-kDa proteins that form tight and stable bundles of actin filaments (Otto et al., 1979; Saishin et al., 1997). Three genetic isoforms are found in

humans, fascin-1, -2 and –3. All of these contain an ABD at their C-terminus and a second unrelated ABS at their N-terminus. Fascin-1 is expressed in most tissues, whilst fascin-2 is expressed in the retina, and fascin-3 expression is largely restricted to the testis (Kureishy et al., 2002). Fascins colocalize with filopodia and it is believed that they are key elements of filopodia formation. The inclusion of fascins with actin in liposomes causes the extension of straight and rigid projections from their surface (Honda et al., 1999).

1.4 Signalling to the actin cytoskeleton

Cells have evolved complex mechanisms to sense and respond to the external environment. Whether keratocytes migrate towards wound areas or neutrophils cross endothelial cells to escape from the blood stream, both need massive re-arrangements of the actin cytoskeleton to perform these tasks (Radice, 1980; Zigmond and Lauffenburger, 1986). Hence, cells can adapt the actin cytoskeleton according to their needs. External signals, such as growth factors, pro-inflammatory molecules or components of the extracellular matrix can bind to membrane receptors that can then lead to the activations of specific signalling pathways. Signalling pathways involve enzymes that catalyze reactions such as (de)phosphorylation and nucleotide exchange. It is possible to distinguish between an input and an output control. The input control determines the temporal and spatial activation of the enzymes. The output control determines the spatial and temporal activation of their downstream partners.

ABPs, which are the major actin-regulatory proteins, are targeted by many cell-signalling proteins and molecules (e.g. PIP_2, Ca^{2+} and PKC) that can activate or inactive them. The activation of many signalling pathways results in Ca^{2+} release and the generation of different types of phosphatidylinositols (PIs). Both tightly regulate

the assembly of actin polymers. Ca^{2+} and PIs can control the activity of capping, monomer-binding and severing proteins. The capping and monomer-binding proteins profilin and CapZ contain a PIP_2 binding site and binding of the phosphoinositide inhibits their association with actin (Heiss and Cooper, 1991). On the other hand, the severing activity of gelsolin can be regulated either by Ca^{2+} or by PIP_2 (Bearer, 1991; Janmey et al., 1987; Yin and Janmey, 2003). Binding of Ca^{2+} to gelsolin activates its severing activity, in contrast to PIP_2, which inhibits gelsolin activity. Also ADF and cofilin activities are inhibited by phosphatidylinositols (Yonezawa et al., 1990). Ca^{2+} and PIs also regulate actin crosslinking proteins. α-Actinin crosslinking activity is enhanced by PIP and PIP_2, and α-actinin-1 and -4 activity is dependent on Ca^{2+} (Fukami et al., 1992; Noegel et al., 1987; Witke et al., 1993). In contrast, filamin actin-crosslinking activity is reduced by PIP and PIP_2 (Furuhashi et al., 1992).

1.4.1 Organization of the actin cytoskeleton by Rho GTPases

The Rho GTPase protein family is known to be a key regulator of signalling pathways that link extracellular and intracellular stimuli to the organization of the cytoskeleton. Signalling through Rho GTPases can be initiated by activation of many different types of plasma membrane receptors (Hall and Nobes, 2000). Rho GTPases are key players in the formation of lamellipodia, membrane ruffles, filopodia and of stable stress fibres. Rho GTPases cycle between an active GTP-bound form and an inactive GDP-bound form. The GTP-bound active form of RhoGTPases activates a plethora of downstream effectors. The cycle between the active and inactive state is regulated by GDP/GTP exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs; Fig. 6). GEFs are responsible for the conversion of the inactive GDP-bound form of the GTPases to the active GTP-

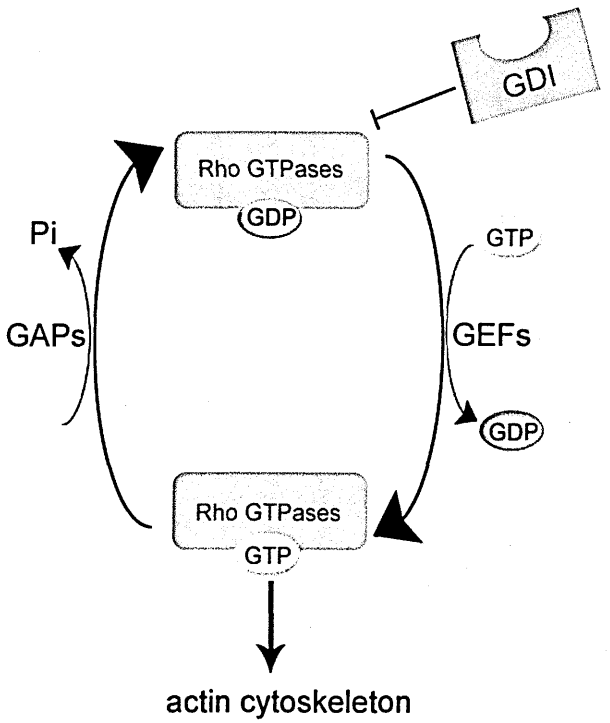


Figure 6. Scheme of Rho GTPase regulation. GDP-bound Rho GTPases are inactive. GDIs inhibit the dissociation of GDP from Rho GTPases. GEFs are able to exchange the GDP for a GTP, thus activating Rho GTPases. In contrast, GAPs enhance the GTPase activity of Rho GTPases.

bound form, and consequently GEFs act as Rho GTPase-signalling activators. GAPs are able to accelerate the low intrinsic GTPase activity of GTPases, thereby GAPs act as Rho GTPase-signalling inactivators. The GDI proteins are capable of inhibiting the dissociation of GDP from Rho GTPases, and thus GDIs also act as Rho GTPase-signalling inhibitors (Schmidt and Hall, 1998).

The Rho family GTPases in mammalian cells include Ras homology (Rho), Ras-related C3 botulinum toxin substrate (Rac) and cell division cycle 42 (Cdc42). Their respective isoforms are RhoA, RhoB and RhoC for Rho, Rac1, Rac2 and Rac3 for Rac and, G25K and Cdc42Hs for Cdc42 (Hall and Nobes, 2000). Rac proteins regulate actin polymerization at the cell periphery to produce lamellipodia and membrane ruffles (Ridley et al., 1992), while Cdc42 proteins are responsible for filopodia formation (Kozma et al., 1995). On the other hand, Rho proteins regulate the assembly of actin stress fibres (Ridley and Hall, 1992). However, locally restricted regulation of actin in specific cell areas is not the result of the activation of the signalling pathway by a single protein but rather the result of the output of several pathways that are activated in parallel. Indeed, Rho family GTPases coordinate cytoskeleton rearrangements in concert and the activation of Cdc42 can lead to activation of Rac (Nobes and Hall, 1995).

Rac and Cdc42 proteins can activate WAVE and N-WASp respectively, which in turn activate the actin nucleation activity of the Arp2/3 complex (Machesky et al., 1999; Miki et al., 1998a; Miki et al., 1998b). Both GTPases can also activate LIM kinase 1 and 2 (LIMK1 and LIMK2), which then phosphorylate and inactivate cofilin, thus inhibiting actin filament disassembly (Arber et al., 1998). LIM kinase carries two LIN-11/ISL1/MEC-3 (LIM) domains, which are important for regulating its kinase activity (Nagata et al., 1999). Rac activation has also been reported to

dissociate gelsolin from the actin filament to promote actin polymerization (Arcaro, 1998), but this interesting possibility has not been confirmed by others.

Rho induces the formation of actin fibres through several downstream effectors including mammalian diaphanous (mDia) and Rho kinase (p160ROCK) (Narumiya et al., 1997; Watanabe et al., 1997). In addition to Rho-mediated stress fibre formation, Rho also induces the formation of focal adhesions through p160ROCK (Narumiya et al., 1997). Focal adhesions are specialized adhesive structures that link the actin cytoskeleton to membrane proteins that bind to the extracellular matrix. p160ROCK is able to active LIMK2, which in turns phosphorylates cofilin. Phosphorylated cofilin dissociates from actin filaments, hence allowing actin polymerization (Maekawa et al., 1999). Although mDia was shown to interact with profilin and to induce actin polymerization, the exact mechanism remains elusive. However it is thought that mDia could recruit profilin to the sites of actin polymerization (Frazier and Field, 1997; Watanabe et al., 1997).

Another effector of Rho family GTPases are PI kinases, which are responsible for the generation of the PIs (e.g. PIP_2 from PIP, and PIP_3 from PIP_2). Rho and Rac physically interact and regulate the PI(4)P 5-kinase that is responsible for the major generation of PIP_2 (Chong et al., 1994; Tolias et al., 1995).

1.5 Contractility of actin filaments

Actin filaments form contractile structures together with myosin and accessory proteins such as tropomyosin, which influence cell division in individual cells or muscle contractility in multicellular organisms. In striated muscle, actin filaments are aligned with myosin filaments in contractile units known as sarcomeres. Based on light microscopy observations, a sarcomere shows a light band (termed I-band

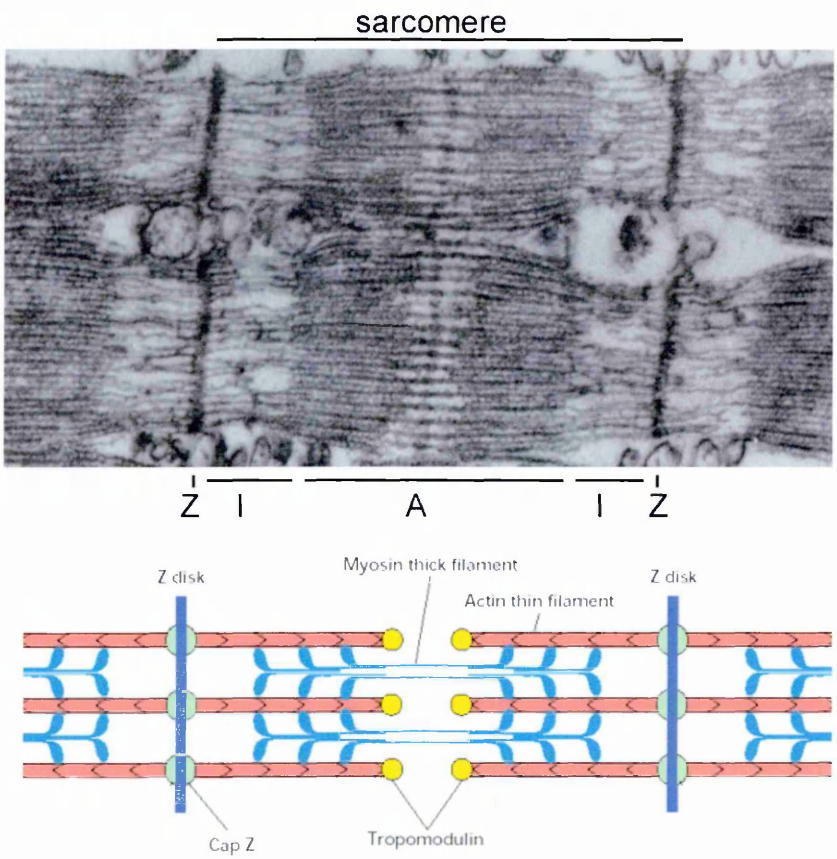


Figure 7. Electron microscopy image of a thin section of rabbit striated muscle (upper panel) and a schematic representation with the main components of the sarcomere (lower panel) (from Huxley, 1957; Lodish et al., 2003). Actin filaments are aligned with myosin filaments in contractile units known as sarcomeres in skeletal and cardiac muscle. Actin thin filaments span the I-band and overlap with myosin thick filaments in the A-band. α-Actinin, CapZ and filamin are found in the Z-discs.

because of its isotropic behaviour under polarized light) and a dark band (termed A-band because of its anisotropic behaviour). Parallel arrays of actin-containing thin filaments span the I-band and overlap with myosin-containing thick filaments in the A-band (Clark et al., 2002). Actin-binding proteins, including α-actinin, CapZ and filamin are found in a structure termed Z-disc, which forms the lateral boundaries of the sarcomeres and where actin filaments are attached through their barbed ends (Fig. 7). Actin filaments start from the Z-discs, span the I-band and extend toward the middle of the sarcomere where they interdigitate with thick filaments in the A-band.

Myosins are motor proteins with the ability to physically displace actin filaments (Umemoto and Sellers, 1990), and they constitute a large superfamily of proteins. Myosins are encoded by 39 genes in humans and are grouped in 15 distinct classes according to their sequence similarities (Coluccio, 2007; Richards and Cavalier-Smith, 2005). Three functional domains are shared among myosins: 1) a motor domain, which interacts with actin and binds ATP; 2) a neck domain, which binds myosin light chains (MLCs); and 3) a tail domain, which is diverse and varies in length and sequence among the myosins (Sellers, 2000). The tail domain serves to anchor and position the motor domain so that in can interact with actin. Myosin II, which is found in muscle and non-muscle cells, is a key player in cell contraction. It is a multimeric protein composed of two heavy chains (MHCs) of ~220-kDa and four MLCs, two of 20-kDa and two of 17-kDa (Lowey et al., 1969; Weeds and Lowey, 1971). Essential MLCs (eMLCs) and regulatory MLCs (rMLCs) are important for the regulation of the myosin motor activity (Clark et al., 2002). Two functional regions can be distinguished in myosin II. The N-terminal region of each MHC and two rMLCs make up the myosin head that forms cross-bridges with the actin filament. The second functional region is formed by the C-termini of the two MHCs, which contain

coiled-coil domains involved in myosin polymerization. The head forms the catalytic motor domain and contains the binding sites for actin and ATP. Actin activates the myosin ATPase activity and the hydrolysis of ATP induces a conformational change of the head that results in displacement of actin filaments (this process results in muscle contraction). The myosin head returns back to its original conformation after the release of ADP (this process results in muscle relaxation). Binding of ATP, its hydrolysis and its release by the head can be performed in a cyclic manner, which in muscle is translated into contraction and relaxation cycles.

In striated muscle, several proteins can regulate the actomyosin-based contraction, which include tropomyosin and troponin (Clark et al., 2002). Tropomyosins (TMs) have a molecular weight of ~37-kDa and are formed by two polypeptide chains arranged as a parallel coiled-coil rod. TMs derive from four genes, TPM1, 2, 3 and 4 in humans and at least 40 isoforms are expressed by alternative splicing (Gunning et al., 2005). TMs form homodimers and heterodimers that cover the length of actin filaments (Tsao et al., 1951). α- and β-TMs are mainly found in striated muscle and form part of the contractile machinery (Helfman et al., 1986). On the other hand, troponin (Tn) is a heterotrimeric complex that is comprised of a Ca^{2+}-binding subunit (TnC), an inhibitory subunit (TnI) and a tropomyosin-binding subunit (TnT) with molecular weights of 18-kDa, 20-kDa and 30-kDa respectively (Westfall and Metzger, 2001). Tn and TM work together to regulate the interactions of thin and thick filaments (Craig and Lehman, 2001). TMs bind along side of actin filaments and block the myosin head-binding sites (Greaser and Gergely, 1971), however, the Tn complex can shift TM's position upon Ca^{2+} binding by TnC, thus unblocking the head-binding sites.

Another important muscle regulatory protein is tropomodulin (Tmod), which has a molecular weight of 40-kDa and regulates actin filament length, organization and contractile activity (Littlefield and Fowler, 1998). Tmod caps the pointed ends of actin filaments *in vitro* and in striated muscle, and its capping activity is enhanced by TM (Weber et al., 1994).

In smooth muscle, although the molecular mechanism of force generation is similar to that in striated muscle, the arrangement of sarcomeres has not been observed (Seow and Pare, 2007). Furthermore, the dynamic of the actin cytoskeleton in smooth muscle cells is similar to that of non-muscle cells (Gerthoffer, 2005). In contrast to striated muscle cells, the actin cytoskeleton of smooth muscle cells is prone to be disassembled by depolymerizing toxins such as latrunculin and cytochalasin. In addition, many of the proteins that control actin dynamics in non-muscle cells are also important for smooth muscle contraction (e.g. Rho GTPases). Another difference between smooth and striated muscle is observed with the components of the contractile apparatus. Although actin, myosin and TM are found in both types of muscles, Tmod and Tn have not been found in smooth muscles cells (Ito et al., 1995; Sobieszek and Bremel, 1975). Furthermore, the contractile apparatus of smooth muscles contains two other regulatory proteins not present in striated muscles. These proteins are caldesmon and calponin (Small and Gimona, 1998).

Caldesmon (CAD) is a 93-kDa protein able to regulate the myosin ATPase activity. While CAD binds actin filaments via its C-terminal domain, it also possesses other domains, which allow the interaction with myosin, TM and the Ca^{2+}-binding proteins calmodulin (CaM) and S100 (Gusev, 2001). The presence of Ca^{2+} and binding of CaM or S100 reverse the inhibitory effect on the ATPase activity of myosin.

Calponin has similar activities to that of CAD. It is a 34-kDa protein and interacts with CaM, S100, myosin, TM and CAD (Graceffa et al., 1996; Szymanski and Tao, 1993; Takahashi et al., 1988; Vancompernolle et al., 1990; Wills et al., 1993; Winder and Walsh, 1990a). Calponin inhibits the actin-activated ATPase activity of myosin and Ca^{2+}CaM is able to inactivate the inhibitory effect of CaP on the ATPases activity of myosin (Abe et al., 1990).

1.6 Calponins

Calponin (CaP) was discovered 22 years ago in an attempt to identify Tn-like proteins in smooth muscles (Takahashi et al., 1988; Takahashi et al., 1986). Cardiac and skeletal muscle contraction/relaxation are tightly regulated by Ca^{2+}, Tn, TM, myosin and actin. Although smooth muscle is also regulated by Ca^{2+} it does not contain Tn. CaP, as well as CAD, were shown to bind actin and to regulate the actin-activated myosin ATPase activity in a Ca^{2+} and CaM dependent manner, thus it was suggested that both could play similar, Tn-like functions in smooth muscles. CaP was shown to share antigenic properties with the C-terminus of TnT. Over the past two decades, CaP has been characterized in greater detail and more is known about its function and its isoforms.

1.6.1 Expression, activation and turnover

In mammals, the three CaP genetic isoforms are transcribed in a cell-type-specific manner. Basic calponin (h1CaP) is the predominant species in smooth muscle cells (SMCs) (Gimona et al., 1990), however it has also been detected in fibroblast, platelets (Takeuchi et al., 1991) and sertoli cells (Zhu et al., 2004). H1CaP is also expressed in other cell types in the context of diverse pathological situations, such as

epithelial cell sarcoma (Yamamura et al., 1998) and mesangial glomerulonephritis (Sugenoya et al., 2002). In tissues and in cultured primary SMCs, h1CaP associates with the central (potentially contractile) actin bundles (Danninger and Gimona, 2000), and it is excluded from cell matrix adhesion sites. Neutral CaP (h2CaP) expression has been detected in both smooth muscle and non-muscle cells (Masuda et al., 1996). In cultured human keratinocytes, h2CaP localizes to the cell-cell junctions, whereas sub-membranous and cytoplasmic accumulation is seen in eccrine sweat glands and myoepithelial cells, respectively. In rat lung alveolar cells, the synthesis and degradation of h2CaP is sensitive to mechanical tension, and prevention of h2CaP degradation requires myosin II-dependent cellular contractility (Hossain et al., 2006). The specific localization of h2CaP around the nuclei of dividing cells and a significant increase in binucleated cells in response to ectopic over-expression of h2CaP suggest its involvement in the regulation of cell proliferation and cytokinesis (Hossain et al., 2003). Over-expression of h2CaP also enhanced endothelial cell migration and wound healing in a zebrafish model (Tang et al., 2006). Expression of acidic CaP (h3CaP) appears mostly restricted to neuronal tissues, although it has also been detected in SMCs and pancreatic cells. In rat brain, h3CaP accumulates specifically in glial cells and astrocytes of the cortex, hippocampus and cerebellum, and in the choroid plexus (Applegate et al., 1994; Ferhat et al., 1996; Represa et al., 1995). The Ca^{2+}-dependent cysteine protease m-calpain cleaves unbound h3CaP *in vitro* (Yoshimoto et al., 2000a).

All CaP variants share a considerable degree of sequence homology throughout their first 273 amino-acids, while they differ completely in their C-terminal regions (Fig. 8). These differences give rise to the variations in molecular

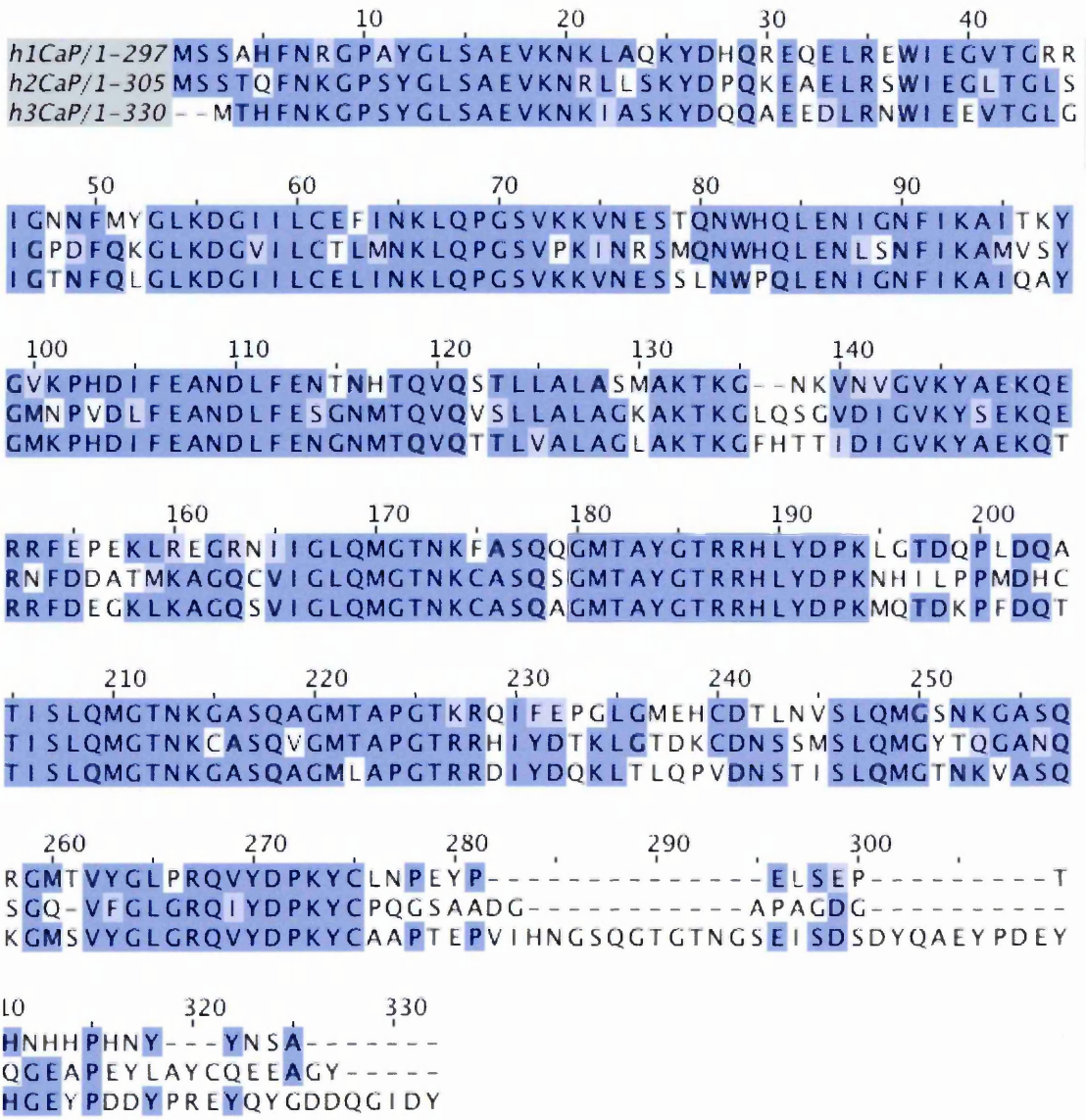


Figure 8. Sequence alignment of the three mouse calponin isoforms used in this thesis. All three CaPs share a high degree of sequence similarity (highlighted in blue) except for their C-terminal tails, which start after Cys275.

Table 1

Isoform	Genbank accession number	Number of amino acids	Molecular weight (Da)	Isoelectric point	Chromosome location	Tissue expression
h1CaP (basic)	BAA04231 Cnn1	297	33,179	8.99	19p13	Smooth muscle
h2CaP (neutral)	Q99439 Cnn2	309	33,697	7.58	19p13 (2, 3, 6, 11, 21)	Most non-muscle cells
h3CaP (acidic)	AAB35752 Cnn3	329	36,413	5.51	1p21	Neurons; glial cells

Chromosome location, basic physicochemical parameters, and tissue expression of the three human CaP genes.

mass and isoelectric points of the CaPs (Table 1), and they also serve as antigenic epitopes for isoform-specific antibodies. The h1CaP molecule is monomeric in solution, and by electron microscopy a variety of contours have been described, ranging from rod shaped to globular, suggesting that there are flexible regions in the molecule (Stafford et al., 1995). Unlike the highly ordered N-terminal portion, structural determination of the complete h1CaP molecule by X-ray crystallography has remained elusive.

In human, mouse and rat, the Cnn1 gene (encoding h1CaP) comprises seven exons that covers 10.2, 10.7, and 8.0 kilobases of genomic sequence, respectively and the human Cnn1 gene was mapped to chromosome 19p13 (Maguchi et al., 1995; Nobrega et al., 2000; Samaha et al., 1996). Fluorescent *in situ* hybridization has indicated that chromosome 1p21 is the most likely site for the Cnn3 gene. The chromosomal location of Cnn2 is ambiguous. While several studies have mapped the Cnn2 gene to chromosome 19 (in close proximity to the Cnn1 gene at p13), others have identified chromosome 21 as its putative gene locus (Cheng et al., 1994; Masuda et al., 1996). Reports of additional assignments to chromosomes 2, 3, 6 and 11 have added to the confusion, and clarification of the exact locus by fluorescent *in situ* hybridization is required to resolve this issue.

1.6.2 Structure

The molecular domain structure of the CaPs as depicted in Figure 9 reveals their modular construction. The most prominent structural element is the N-terminal CH domain, a predominantly helical globular structure that gave name to a large superfamily of protein-protein interaction modules present in many actin filament crosslinkers and bundlers. The second modular component is the short tandem

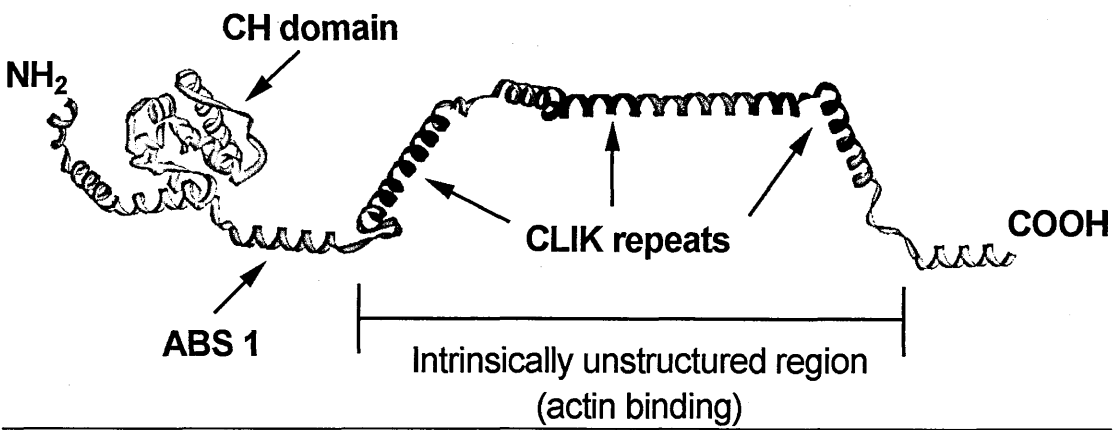


Figure. 9. Schematic representation of the CaP molecule. All three CaPs share a similar molecular domain organization. Following the hydrophilic Ca2+/CaM-binding peptide the highly structured N-terminus is dominated by the 100 residue long, globular CH domain, and an adjacent, surface-exposed region that contains the myosin ATPase inhibiting peptide, and actin-binding site 1 (ABS1). The tandem CLIK[23] repeats constitute the major actin-binding site in CaPs. They remain unfolded in solution and appear to structurally reorganize upon contact with the actin filament. All putative phosphorylation sites (Ser175, 215, 254; Thr170, 180, 184, 220, 224, 259; Tyr182 and Tyr261) lie within this region. The C-terminal end contains the tail regions that are unique in length and composition in all three CaP isoforms.

sequence repeats in the C-terminal half. Both the highly basic, 23-residue repeat sequences, and the acidic intervening sequences follow the consensus of a type I intrinsically unstructured protein, which may account for the flexible folding properties of the CaP molecules. Between the CH domain and the tandem repeats there is a region spanning amino-acids142-163 that forms an ABS, which mediates actin binding and that contains the myosin ATPase inhibitory peptide (residues 142-147) (el-Mezgueldi et al., 1996; Mezgueldi et al., 1992). The extreme C-termini of CaPs contain the isoform-specific tail sequences. The CH domains show high functional plasticity (Gimona et al., 2002). Documented interactions with CH domains range from actin, microtubules and intermediate filaments, to mitogen-activated protein kinases (MAPKs), Zn^{2+} fingers, Ca^{2+}-binding proteins of the S100 family, and phospholipids. The involvement of the CH domain of CaPs in actin binding, however, remains controversial. Although earlier cryoelectron microscopy analysis placed the h1CaP CH domain into the large groove of the actin filament, deletion studies have shown that the CH domain is neither sufficient nor necessary for thin filament binding, and that instead, the C-terminal tandem repeats mediate the CaP-actin association (Gimona and Mital, 1998; Hodgkinson et al., 1997). This view is supported by evidence for a CH domain-like mass at the putative CH domain location, using a recombinant h1CaP deletion construct that lacks the CH domain (Galkin et al., 2006). The calponin like ($CLIK^{23}$) repeats are essential for the actin-binding function of the CaPs, and the strength of actin binding is directly correlated to the number of $CLIK^{23}$ repeats. *In vitro* F-actin co-sedimentation assays using recombinant proteins, and over-expression studies in cultured cells have shown that the mode of binding and the binding interface are conserved in CaP-like molecules from worms to man. The binding of multiple $CLIK^{23}$ repeats reduces actin filament

turnover in living cells and strongly interferes with cytokinesis (Lener et al., 2004). Comprehensive deletion and domain swap experiments have revealed that the unique tail sequences of all three CaP isoforms negatively regulate their F-actin binding (Burgstaller et al., 2002).

The presence of one or multiple CLIK[23] repeats identifies the members of the CaP family. All vertebrate CaPs are constructed following the principles depicted in Figure 9. Vertebrates also express CaP-related molecules of the smooth muscle protein 22 (SM22)/Transgelin (TGN) family, which contain single copies of the CLIK[23] motif. The three genetic SM22/TGN variants (TGN1, TGN2 and TGN3/NP25) exhibit similar basic modular arrangements, and cell-type-specific expression. Invertebrate orthologues of the CaP-related proteins are found in the tapeworm *Echinococcus granulosus* (myophilin) (Martin et al., 1995) and in *Drosophila melanogaster* (mp20) (Ayme-Southgate et al., 1989). The trematode parasites *Schistosoma mansoni* and *japonicum* express a unique CaP homologue that contains five complete CLIK[23] modules (Yang et al., 1999). Nematodes, including the *Caenorhabdites* family, and the parasite *Onchocerca volvulus* lack close CaP homologues, although they contain an SM22/TGN homologue (incorrectly annotated as a CaP homologue, Cpn-1). Notably, UNC-87 (in *C. elegans*) and OV9M (in *O. volvulus*) each contain seven copies of the CLIK[23] motif, but do not contain an N-terminal CH domain (Goetinck and Waterston, 1994; Irvine et al., 1994). UNC-87 is a component of the worm muscle thin filaments and shows actin binding and bundling activity *in vitro*. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* lack any of the multiple-CLIK[23] repeat components. Scp1 (*S. cerevisiae*) and SPAC4F8.10c (*S. pombe*) resemble single CLIK[23]-containing SM22/TGN, but functional data exist only for Scp1(Winder et al., 2003); Scp1 binds and bundles actin filaments in-vitro.

1.6.3 Biological functions

In vitro, all CaPs bind to both muscle and non-muscle actin, although it is currently not clear if *in vivo* the CaP isoforms have a preference for any of the six mammalian actin isoforms. At low CaP to actin ratios, CaP binds to F-actin via a globular motif that comprises its entire C-terminal region (including the ABS1, the tandem CLIK[23] repeats, and the regulatory tail) (Galkin et al., 2006). Native h1CaP from chicken gizzard binds to immobilized G-actin with a similar affinity to F-actin (Kd, 0.15 mM) (Ferjani et al., 2006b). The hypothesis that G-actin regulates the activity status of CaP is intriguing, but it requires confirmation at the cellular level. The binding of CaP to F-actin is sensitive to CaP phosphorylation at several Ser, Thr and Tyr residues that are located in the C-terminal half of the molecule (Abouzaglou et al., 2004; Nakamura et al., 1993; Winder et al., 1993a; Winder and Walsh, 1990b). The binding of Ca^{2+}/CaM to h1CaP (but not to h3CaP) inhibits the interaction with actin, and consequently releases the block on actin-activated myosin MgATPase activity (Abe et al., 1990; Applegate et al., 1994; Mezgueldi et al., 1992).

One Ca^{2+}/CaM-binding site (amino-acids 1-30) resides in the N-termini of the CaPs and a second site exists just before ABS1. Information on potential CaP interactions with microtubules and intermediate filaments (vimentin or desmin) has come from in-vitro biochemical studies and localization studies in smooth muscle by electron microscopy (Fujii et al., 1997; Fujii and Koizumi, 1999). Binding to the desmin tetramer subunit is stronger than to the polymerized filament, suggesting the possible involvement of CaP in the assembly mechanism of intermediate filaments (Fujii et al., 2000; Mabuchi et al., 1997; Wang and Gusev, 1996). Additional interactions with components of the cytoskeleton have been described for TM (Vancompernolle et al., 1990), myosin (Szymanski and Tao, 1993), CAD (Graceffa et

al., 1996), smooth muscle archvillin (Gangopadhyay et al., 2004), the S100 proteins (Wills et al., 1993), and gelsolin (Ferjani et al., 2006a). CaP also interacts with signalling components, including ERK1/2 (Menice et al., 1997), p160ROCK (Kaneko et al., 2000), PKCα and PKCε (Leinweber et al., 2000). ROCK phosphorylates CaP at several sites (Thr170, Ser175, Thr180, Thr184 and Thr259), which provides another regulatory mechanism for CaP, in addition to the phosphorylation by Ca^{2+}/CaM kinase II (CaMKII), and PKCα. In addition, CaP also was found in a cGMP kinase signalling complex involved in the regulation of the Ca^{2+}-ATPase II (SERCA) activity together with the IP_3 receptor I (IP_3RI), IP_3 receptor associated cGMP kinase substrate (IRAG) and cGMP kinase 1β (Koller et al., 2003). Since most of these data have come from experiments with h1CaP, the general validity of these mechanisms towards all of the CaP isoforms awaits experimental verification.

In knock-out mice lacking h1CaP, SMC contraction and Ca^{2+}-sensitivity is marginally affected, with the exception of an increase in unloaded shortening velocity of these cells, which indicates a negligible involvement of h1CaP in mediating Ca^{2+}-dependent contractile signals (Matthew et al., 2000; Yoshimoto et al., 2000b). Notably however, these animals have a significant reduction in actin and TM expression, indicating a relationship between the expression levels of h1CaP and transcription of the components of the actin machinery. Loss of h1CaP leads to enhanced ectopic bone formation *in vivo*, which is mediated by bone morphogenetic protein in h1CaP knock-out mice, suggesting that h1CaP negatively regulates the osteogenic programme (Yoshikawa et al., 1998).

1.6.4 Differentiation and pathology

Growth factors regulate the expression levels of h1CaP in SMCs. Up-regulation of h1CaP, in response to transforming growth factor (TGF)-β1, is in line with a general differentiation-promoting activity (Ueki et al., 1998). Stimulation by tumor necrosis factor (TNF)-α and platelet-derived growth factor (PDGF)-BB almost completely abrogates h1CaP expression in cells cultured on glass surfaces, while aortic vascular SMCs cultured on polymerized collagen require both PDGF-BB and interleukin (IL)-1β to alter h1CaP expression (Chen et al., 2006; Dykes and Wright, 2007). It will be important to determine if expression of the other CaP isoforms is similarly sensitive to growth-factor-induced signalling, and how the composition and mechanical properties of the extracellular matrix modulate the conductivity for transcriptional regulation.

Together with less than a handful of components, the expression of h1CaP serves as a reliable marker for the differentiated contractile smooth muscle phenotype. Dedifferentiated SMCs that convert to the synthetic phenotype, and primary SMCs in culture rapidly down-regulate h1CaP expression. In response to activin-A-stimulated, and actin responsive element (ARE)-mediated transcriptional activation, pancreatic AR42J-B13 cells (which expresses all three CaP isoforms) down-regulate h1CaP expression and produce insulin, whereas over-expression of h1CaP suppresses activin-A-induced ARE activity and differentiation (Morioka et al., 2003). The CH domain is necessary for this suppression, and it was thus proposed that a specific function that resides in the CH domain of h1CaP (but not of h2CaP or h3CaP) regulates pancreatic cell differentiation and transcriptional activity. Over-expression of h3CaP in cultured rat hippocampal neurons increases the density and length of dendritic spines, indicating a regulation of neuronal cell plasticity by h3CaP (Ferhat et

al., 2003). This effect requires the actin-binding domain of h3CaP, in agreement with an actin filament-stabilizing function of CaPs.

Expression of h2CaP regulates endothelial cell migration both *in vitro* and *in vivo*, and h2CaP is involved in guiding vascular development through its regulation of ERK signalling, by increasing ERK phosphorylation levels via MAPK (Tang et al., 2006). Differential gene expression profiling of solid versus metastatic adenocarcinoma has revealed a molecular signature that comprises 17 genes (Ramaswamy et al., 2003). Down-regulation of h1CaP expression as part of this signature profile predicts, with high fidelity, the propensity of a solid tumour to form metastases. Independent studies on osteosarcoma (Yamamura et al., 1998) and fibrosarcoma (Takeoka et al., 2002) have confirmed the importance of h1CaP expression in the suppression of tumorigenesis and metastasis . Subsequent investigations *in vitro* in metastasizing adenocarcinoma and melanoma cell lines have revealed that elevated expression levels of h1CaP indeed reduce cell motility and the formation of invasive podosome adhesions (Gimona et al., 2003). The actin-binding CLIK^{23} repeats are the necessary and sufficient modules for mediating this activity (Lener et al., 2004). In further support of a tumour suppressing function, the loss of h1CaP prevents neovascularization of tumours and regulates the maturation of blood vessels, also in the tumour-host microenvironment (Yamamura et al., 2007). Ectopic expression of h1CaP reduces tumour size, DNA synthesis, mitotic activity, cell motility, and suppresses proliferation and tumorigenesis in human leiomyosarcoma. As a bona fide tumour suppressor, h1CaP has thus considerable potential for gene-therapy (Takahashi and Yamamura, 2003). In addition, h1CaP could be related to many other human diseases where the etiologies are still unknown. The chromosome region 19p13.2 where h1CaP resides has been associated with tumour progression

(Koga et al., 2002a; Koga et al., 2002b; Pezzolo et al., 2008; Prazeres et al., 2007; Stankov et al., 2004; Yang et al., 2004), with systemic lupus erythematosus (Namjou et al., 2002), Alzheimer disease (Wijsman et al., 2004), hearing impairment (Santos et al., 2006) and polycystic ovary syndrome (Urbanek et al., 2005).

1.7 CH domains

The ABD of many actin crosslinkers consists of two CH domains in tandem. CH domains are ~100 amino acids long and with the refinement of algorithms for the identification of sequence motifs, more CH domain-containing proteins were identified. Currently, CH domains have been identified in tandem in ABPs and as single domain in many ABPs and signalling proteins (Stradal et al., 1998). Six CH domain subtypes are distinguished according to their sequence similarities. Type 1 and type 2 CH domains are generally found in tandem and form an ABD, although type 2 CH domains have been also found as a single domain (e.g. smoothelin). Type 3 CH domains are present in ABPs (e.g. calponin and SM22α) and signalling proteins (e.g. Vav, IQGAP and ARHGEF6). Types 4 and 5 CH domains have been found in tandem in the focal adhesion-associated protein parvin. Both CH domains are phylogenetically closer to the type 1 CH domain. Type 6 CH domains are found in the end-binding (EB) protein family and are thought to mediate microtubule binding (Gimona et al., 2002).

The overall secondary and tertiary structures of the CH domains seems to be conserved, although CH domain subtypes show low sequence similarities, which could allow functional diversity among the CH domains. The ABDs are able to bind actin filaments but only the type 1 CH domains have the ability to interact with filaments, while the type 2 CH domains do not (Way et al., 1992). Yet, they

contribute to the actin-binding affinity of the whole ABD (Lorenzi and Gimona, 2008).

1.7.1 Type 3 CH domains

Type 3 CH domains are found as a single module in cytoskeletal and signalling proteins such as CaP, SM22α or TGN, the protoncogene Vav (Katzav et al., 1989), IQ motif containing GTPase activating protein (IQGAP) and Rho guanine nucleotide exchange factor 6 (ARHGEF6). Type 3 CH domains lack actin-binding activity and their biological function in many proteins remains elusive. It was shown for Vav that the CH domain regulates its GEF activity (Aghazadeh et al., 2000; Zugaza et al., 2002). Vav is a 98-kDa protein that regulates the GTPase activity of the Rho family GTPases facilitating the exchange of GDP for GTP (Olson et al., 1996). Vav activation is regulated by tyrosine phosphorylation (Crespo et al., 1997) and it is thought that a deletion in the CH domain is the cause for its transforming activity (Katzav et al., 1991) mediated by a phosphorylation-independent GEF activity. The exchange activity of Vav is autoinhibited by the intramolecular binding of the acidic region (Ac) and the Dbl homology (DH) domain where the GEF activity resides. This interaction is interrupted upon tyrosine phosphorylation of the protein and Vav becomes active (Aghazadeh et al., 2000). However, the deletion in oncogenic Vav seems to impair the contact between the Ac region and the DH domain (Hornstein et al., 2004).

IQGAP is a protein of 189-kDa, which carries an N-terminal type 3 CH domain and binds actin filaments directly (Bashour et al., 1997), but the actin-binding site has not yet been defined precisely. IQGAP also possesses several IQ domains that bind to CaM (Hart et al., 1996) and a Ras GTPase-activating protein (GAP)-related

domain; however it does not show GAP activity. IQGAP is able to integrate signalling pathways with the actin cytoskeleton, since actin-regulatory proteins such as Cdc42 and Rac1 interact directly with IQGAP (Kuroda et al., 1996).

ARHGEF6 or αPIX is a 61-kDa protein and like Vav it also possesses GEF activity. Interestingly the N-terminal CH domain of ARHGEF6 seems to be functionally similar to the CH domain of Vav, and thus ARHGEF6 lacking the CH domain shows constitutive GEF activity (Daniels et al., 1999). In addition, mutations on the CH domain have been associated to X-linked forms of mental retardation (Kutsche et al., 2000).

SM22α, which resembles a shorter version of CaP carries an N-terminal CH domain followed by a single $CLIK^{23}$ repeat, which contains the actin-binding activity of the protein (Gimona and Mital, 1998). Recently it was shown that SM22α is able to regulate the expression of the 92-kDa gelatinase (MMP-9) protein, which is involved in matrix degradation and cancer cell invasion. Even more, it was demonstrated that the presence of the CH domain is required for MMP-9 downregulation (Nair et al., 2006).

The type 3 CH domains of calponins have been shown to be important for the differentiation of pancreatic acinar cells into insulin producing cells (Morioka et al., 2003) and have been shown to interact with ERK and PKC (Leinweber et al., 2000; Leinweber et al., 1999). However, the physiological roles of these interactions remain elusive.

1.8 Cell division

Contractile networks of actin filaments are fundamental for cell division. In combination with ABPs (e.g. cortexillin, fimbrin and myosin), actin filaments tune the

mechanical properties of the actin cytoskeleton to constrict the mother cell into two daughter cells. The life-cycle of cells are separated into four major phases known as G1, S, G2 and mitosis (M phase). During G1, S and G2 cells prepare all of the molecular machinery necessary to go through M phase including DNA duplication (S phase) (Lodish et al., 2003).

The life-cycle of cells and the molecular processes involved during the many phases of cell division differ among organisms. Eukaryotic cell division is different from prokaryotic cell duplication, and within the eukaryotic world differences are found between plants and animals. The major differences between plant and animal cell division are found in the cytokinesis process during the last phase of mitosis, which is responsible for partitioning the cell components into the two daughter cells. The process of mitosis can be divided into several stages known as prophase, pro-metaphase, metaphase, anaphase and telophase. Chromosomes are condensed into chromatids during the prophase. Prometaphase is characterized by the break down of the nuclear envelope and the formation of microtubule-based structure known as the mitotic spindle apparatus. During metaphase chromosomes are positioned on the spindle equator of cells with the help of the spindle microtubules. Anaphase is characterized by the beginning of cytokinesis and the movement of chromosomes to opposite poles of cells (Lodish et al., 2003).

Cytokinesis is driven by a specialized structure known as the contractile ring, which is close to the plasma membrane that creates a cleavage furrow that divides the two daughter cells. The major steps of cytokinesis are cleavage furrow formation at cell periphery, furrow ingression, midbody formation and cell separation. During cleavage furrow ingression the central microtubules get compressed and form a compact structure known as the midbody. This structure is not yet understood in

detail. The last step of cytokinesis is cell separation, and this occurs by abscission, which was recently described to be a process that requires lipid transport that allows the physical separation of the daughter cells (Matheson et al., 2005).

1.8.1 Role of actin-binding proteins during cytokinesis

The contractile ring is an actomyosin-based structure (Fujiwara et al., 1978; Schroeder, 1973), which generates the required forces to promote furrow ingression (Mabuchi and Okuno, 1977; Yamakita et al., 1994). As a general view, myosin II pulls actin filaments that are either crosslinked to other actin filaments or to the plasma membrane constricting the cleavage furrow cortex (Reichl et al., 2008). Myosin II is crucial for cytokinesis progression and phosphorylation is necessary for its activation and for force generation (Satterwhite et al., 1992; Yamakita et al., 1994). During cytokinesis, myosin II can be regulated by Rho family GTPases (Matsumura, 2005). It is known that RhoA activates ROCK, which in turn phosphorylates MLC, thus participating in the activation of myosin II. On the other hand, myosin II can be activated by myosin light chain kinase (MLCK), but it is not known what activates MLCK during cytokinesis (Matsumura, 2005). Strikingly, it is not yet fully understood how myosin and actin-crosslinkers interact to control the dynamics of furrow ingression. It is thought that actin-crosslinking proteins are necessary at the contractile ring to arrange the cortical actin filaments in a manner such that myosin can pull and exert tension on the plasma membrane. Overexpression of α-actinin leads to aberrant cytokinesis due to inhibition of actin filament turnover (Mukhina et al., 2007), while depletion of α-actinin induces accelerated cytokinesis. The actin filament-crosslinker cortexillin has also been found to be necessary for cytokinesis progression. Depletion of cortexillin leads to the formation of giant binucleated cells

(Faix et al., 1996). Currently, cortexillin is one of the few actin-crosslinking proteins that have a direct role in cytokinesis. Depletion of most of the actin crosslinkers does not lead to significant defects in cell division (Wu et al., 2001); on the other hand, their overexpression does. The fact that depletion of actin crosslinkers does not affect cell division might be explained by the overlapping functions of actin-crosslinking proteins.

Aim of the study

The actin-binding activity of h1CaP has been extensively studied *in vitro* and it has been demonstrated that its affinity for actin filaments is reduced by Ca^{2+}/CaM, phosphorylation and its C-terminal tail. However, little is known about its regulation in live cells, and whether these regulatory mechanisms occur *in vivo* remains unknown. The aim of this study was to describe and understand the regulatory mechanism that controls h1CaP actin-binding function in live cells, and to investigate the role of h1CaP during cell division.

CHAPTER 2: MATERIAL AND METHODS

2.1 Reagents and antibodies

Mouse monoclonal antibody against Myc tag was purchased from Santa Cruz (Santa Cruz, CA) and against His tag from QIAGEN (Milano, Italy). Rabbit polyclonal antibody against GST was a gift from Dr. Antonella DeMatteis (Consorzio Mario Negri Sud). Horseradish peroxidase conjugated goat antibody against mouse antibody was purchased from Amersham (Milano, Italy). Mouse monoclonal antibody against basic calponin was purchased from Dako (Milano, Italy). Phalloidin labelled with Alexa 568 was from Molecular Probes (Milano, Italy). HiTrap chelating HP and glutathione sepharose 4 fast flow from Amersham (Milano, Italy) were used to purify His and GST tagged proteins, respectively. All other reagents used in this study were either obtained as specified elsewhere, or were obtained at the highest possible purities from Sigma Aldrich and Calbiochem.

2.2 Cell culture, transfection and fluorescence microscopy

A7r5 rat vascular smooth muscle cell line (ATTC) were grown in low glucose Dulbecco's Modified Eagle's Medium (Invitrogen, Milano, Italy) supplemented with 10% foetal bovine serum (PAA, Linz, Austria), penicillin/streptomycin/glutamine (Invitrogen, Milano, Italy) at 37 °C and 5% CO_2. For transient transfections, cells were grown in 60 mm plastic culture dishes and transfected with Superfect (QIAGEN, Milano, Italy) at 70% confluence, essentially as described elsewhere (Kranewitter et al., 2001). Cells were replated onto 15 mm cover slips 16 h post-transfection and prepared for fluorescence microscopy. For staining, cells were washed three times in phosphate-buffered saline (PBS) (138 mM NaCl, 26 mM KCl, 84 mM Na_2HPO_4, 14

mM KH_2PO_4, pH 7.4), fixed in 3.7% paraformaldehyde (PFA; Merck) in PBS for 30 min and permeabilized in 3.7% PFA / 0.3% Triton X-100 in PBS for 1 min. Fluorescence images were recorded on a Zeiss Axioscope equipped with an Axiocam driven by the manufacture's software package equipped with a 63x, 1.4NA Plan-APOCHROMAT objective (Zeiss). Live images of transiently transfected A7r5 cells with GFP constructs were recorded on a Nikon Eclipse TE2000 equipped with a 60x Plan Apo VC 1.40NA ∞/0.17 WD 0.13 objective (Nikon).

2.3 Vectors and constructs

Figure 10 shows a schematic representation of the constructs used in this study and Table 2 lists the oligonucleotide sequences of the primers used to generate the constructs. Dr. Mario Gimona (Consorzio Mario Negri Sud) has kindly provided the pEGFP mammalian expression vectors harbouring the cDNAs of the mouse basic, neutral and acidic calponin, and a tail-deleted form of basic calponin. The pEGFP mammalian expression vectors harbouring the cDNAs of the mouse basic calponin and the tail deleted form (Danninger and Gimona, 2000) were used as templates and amplified by PCR (using PFU Turbo, Stratagen) with the primer pairs 0001/0055 and 0001/0068, respectively, and subcloned into the BspEI/EcoRI-digested pEGFP-C1 (BD Biosciences/Clontech) expression vectors. pEGFP-C1 harbouring the sequence of basic calponin was further used as template for the generation of h1CaP lacking CH domain (h1CaPΔCHD) using the primer pairs 00135/0006 and sublcloned into BspEI/BamHI-digested pEGFP-C1 expression vector. pEGFP-C1 harbouring the sequence of the tail-deleted form of basic calponin was used as template for the generation of h1CaP lacking the CH domain and the C-terminal tail (h1CaPΔCHDΔT) using the primer pairs 0135/0068 and sublcloned into BspEI/EcoRI-digested pEGFP-

C1 vector. The cDNA of h1CaP in pEGFP-C1 was also used as a template for the amplification of the CLIK[23] repeats with (CLIK) and without the C-terminal tail (CLIKΔT) using the primer pairs 0050/0055 and 0050/0068, respectively, and both have been subcloned into BspEI/EcoRI-digested pEGFP-C1 expression vectors. pEGFP carrying the genes for basic calponin with a S175A (h1CaP-S175A) and S175D (h1CaP-S175D) mutations was obtained from Dr. Gimona. These constructs were used as templates for the amplification of both mutant calponins using the primer pairs 0001/0055 and subcloned into BspEI/EcoRI-digested pEGFP-C1. pEGFP-C1 vectors harbouring the sequences for h1CaP-S175A and h1CaP-S175D were used as templates for the generation of an additional point mutation at Ser254 by site directed mutagenesis (Quickchange, Stratagene) using the primer pairs 0062/0063 for the generation of S254A (h1CaP-S175/254A) and 0064/0065 for the generation of S254D (h1CaP-S175/254D). The products of the site directed mutagenesis have been subcloned into a new BspEI/EcoRI-digested pEGFP-C1 expression vector.

The h1CaP mutant lacking the helix A and loop 1 (h1CaPΔHxAΔL1) was generated in the following way: the pEGFP-C1 vector harbouring the cDNA of basic calponin was used as template for the amplification of two fragments; one corresponding to the nucleotide positions 1 to 142 (Frg1) and a second fragment (Frg2) corresponding to the nucleotides 169 to 894 using the primer pairs 0001/0117 and 0121/0006, respectively. Frg1 and 2 were digested with the endonuclease restriction enzymes BspEI/PvuII and DraI/BamHI, respectively. The two fragments were then subcloned into BspEI/BamHI-digested pEGFP-C1 expression vector.

pEGFP-C1 harbouring the cDNA of basic calponin was used as a template and amplified by PCR with the primer pairs 0083/0084 and subcloned into the EcoRI/XhoI- digested pCMV-Myc mammalian expression vector (Clontech).

The Myc tagged h1CaP mutant lacking the helix A and loop 1 (h1CaPΔHxAΔL1) was generated in the following way: pCMV-Myc vector harbouring the cDNA of basic calponin was used as template for the amplification of two fragments; one corresponding to nucleotides 1 to 142 (Frg1) and a second fragment (Frg2) corresponding to the nucleotides 169-894 using the primer pairs 0083/0117 and 0121/0084, respectively. Frg1 and 2 were digested with the endonuclease restriction enzymes EcoRI/PvuII and DraI/XhoI, respectively. The two fragments were then subcloned into EcoRI/XhoI-digested pCMV-Myc expression vector.

The pEGFP vector harbouring the cDNA of basic calponin (Danninger and Gimona, 2000) was used as a template and the nucleotide sequence encoding for the CH domain was amplified by PCR with the primer pairs 0042/0043 and 0024/0044 and subcloned into the NdeI/XhoI-digested pET-22b (Novagen) and XhoI/BamHI-digested pGEX-4T2 (Amersham) bacterial expression vectors, respectively.

The C-terminal tail of h1CaP fused to GST and Myc (GST-tail-myc) was generated in the following way: The pEGFP-C1 vector carrying the cDNA of basic calponin was used as a template and the nucleotide sequence encoding for the C-terminal tail was amplified by PCR with the primer pairs 0071/0072 and subcloned into EcoRI/BamHI-digested pTRChis2b (Invitrogen) bacterial expression vector. The pTRChis2b vector harbouring the nucleotide sequence of the tail with a Myc tag was amplified by PCR and subcloned into BamHI/SalI-digested pGEX-4T2 bacterial expression vector.

pEGFP mammalian expression vectors harbouring the cDNA of the mouse SM22α and the human Vav1 was provided by Dr. Gimona. The recombinant h1CaP carrying the CH domain of SM22α (h1CaP-SMCH) or Vav1 (h1CaP-VavCH) was

generated in the following way: pEGFP-C1 vector harbouring the cDNA of basic calponin was used as template for the amplification of two fragments; one corresponding to the nucelotides 1 to 78 (F1) and a second fragment (F2) corresponding to the nucleotides 403-894 using the primer pairs 0001/0045 and 0048/0006, respectively. pEGFP carrying the cDNA of SM22α was used as a template for the amplification by PCR of a third fragment (F3) corresponding the nucleotide sequence encoding for the CH domain of SM22α using the primer pairs 0003/0004. Frg2 and 3 were first 5'-phosphorylated with T4 polynucleotide kinase (Invitrogen) and then digested with the endonuclease restriction enzymes BamH1 and SacI, respectively. The two fragments were then subcloned into SacI/BamHI-digested pEGFP-C1 expression vector. Then F3 was digested with the restriction enzymes BspEI/SacI and subcloned into BspEI/SacI-digested pEGFP-C1 vector harbouring the sequences of F2 and F3. The recombinant h1CaP-VavCH was generated in a similar manner with the following differences: F2 was generated with the primer pairs 0049/0006 and F3 was amplified by PCR from pEGFP carrying the cDNA of Vav1 using the primer pairs 0034/0032.

The recombinant h1CaP with and without the tail fused to a N-terminal citrine and a C-terminal CFP (Y-h1CaP-C and Y-h1CaPΔT-C) were generated in the following way: The pEGFP-C1 vector carrying the cDNA of basic calponin was used as a template and the nucleotide sequence encoding for the full-length protein or a tail-deleted form was amplified by PCR using the primer pairs 0100/0099 and 0095/0096, respectively. Dr. Klaus M. Hahn (California, USA) kindly provided a RhoA biosensor, which was used as a template to amplify by PCR the nucleotide sequence coding for the citrine with the primer pairs 0093/0094. Then the PCR products of full-length calponin and the tail-deleted form were digested with the

endonuclease restriction enzymes XhoI/EcoRI, and the citrine nucleotide sequence with EcoRI/BamHI. The citrine was then subcloned either with the full-length calponin or with the tail-deleted form into a XhoI/BamHI-digested pCDNA4a (Invitrogen) mammalian expression vector. The pECFP (BD Biosciences/Clontech) mammalian expression vector was used as a template and the nucleotide sequence coding for the CFP was amplified by PCR with the primer pairs 0093/0094. The PCR product was digested with the restriction enzymes XhoI/ApaI and subcloned into XhoI/ApaI-digested pCDNA4a vectors harbouring the nucleotide sequence coding for the full-length calponin or the tail-deleted form fused to a citrine.

CODE	SEQUENCE 5′ → 3′
0001	GCTTCCGGAATGTCTTCTGCACATTTTAAC
0003	GACGAGGAGCTCGAGGAGCG
0004	TTTGGTCACAGCCAAACTGCC
0006	CCAGGATCCCTAGGCAGAGTTGTAGTAGTTGTGC
0024	CCCGGGATCCGACCATCAGCGGGAGCAGG
0032	CTGGGCGATCGGGGTCCAGG
0034	GGAATTCGAGCTCTGGCGCCAATGCACCC
0042	GGAATTCCATATGGACCATCAGCGGGAGCAGG
0043	GAGTGACGCTCGAGTTTGTCTTGGCCATGCTGGCC
0044	GCTGATGCCTCGAGTCATTTGTCTTGGCCATGCTGGCC
0045	CTTAAGGGAGCTCCTCGTCGTATTTCTGGGC
0048	GGAAACAAAGTCAATGTGGGAGTC
0049	GGAAACAAAGTCAATGTGGGAGTCAAG
0050	GGCAGGTCCGGAATTGGACTGCAGATGGGCAC
0055	GCGAATTCTAGGCAGAGTTGTAGTAGTTGTGCGG
0062	GGGGCCGCACAGAGGGGCATGACAGTGTATGGGCTTCC
0063	GGAAGCCCATACACTGTCATGCCCCTCTGTGCGGCCCC
0064	GGGGCCGACCAGAGGGGCATGACAGTGTATGGGCTTCC
0065	GGAAGCCCATACACTGTCATGCCCCTCTGGTCGGCCCC
0068	CGGAATTCCTATGCGCAGTACTTGGGATCGTACACC
0071	GCGGATCCGCTGAACCCGGAGTACCCAGAG
0072	CGAATTCTGGCAGAGTTGTAGTAGTTGTG
0078	GGATCCCTGAACCCGGAGTACCCAGAG
0083	GGAATTCGGATGTCTTCTGCACATTTTAACC
0084	CCGCGATCCTCGAGCTAGGCAGAGTTGTAGTAGTTGTGC
0084	CCGCGATCCTCGAGCTAGGCAGAGTTGTAGTAGTTGTGC
0093	CGGGATCCATGGTGAGCAAGGGCGAGGAG
0094	GTCCGAATTCTCCGGACTTGTACAGCTCGTCCATGC
0095	CAGCGAATTCATGTCTTCTGCACATTTTAACCG
0096	CGAGTTCGCTCGAGGGCAGAGTTGTAGTAGTTGT
0099	GTCGACGGGCCCCTACTTGTACAGCTCGTCCATGCC
0100	GGAGTTCGCTCGAGTCCGGAATGGTGAGCAAGGGCGAGGAGCTG
0117	CGGCGGCAGCTGGGCCAGCTTGTTCTTTACTTCAGC
0121	CCCCGGTTTAAATACGGGATCATTCTTTGCG
0135	GCCAAGTCCGGAGGAAACAAAGTCAATGTGG

Table 2. List of primers to generate the constructs used in this study. The code is an internal number used in the laboratory to track the sequences.

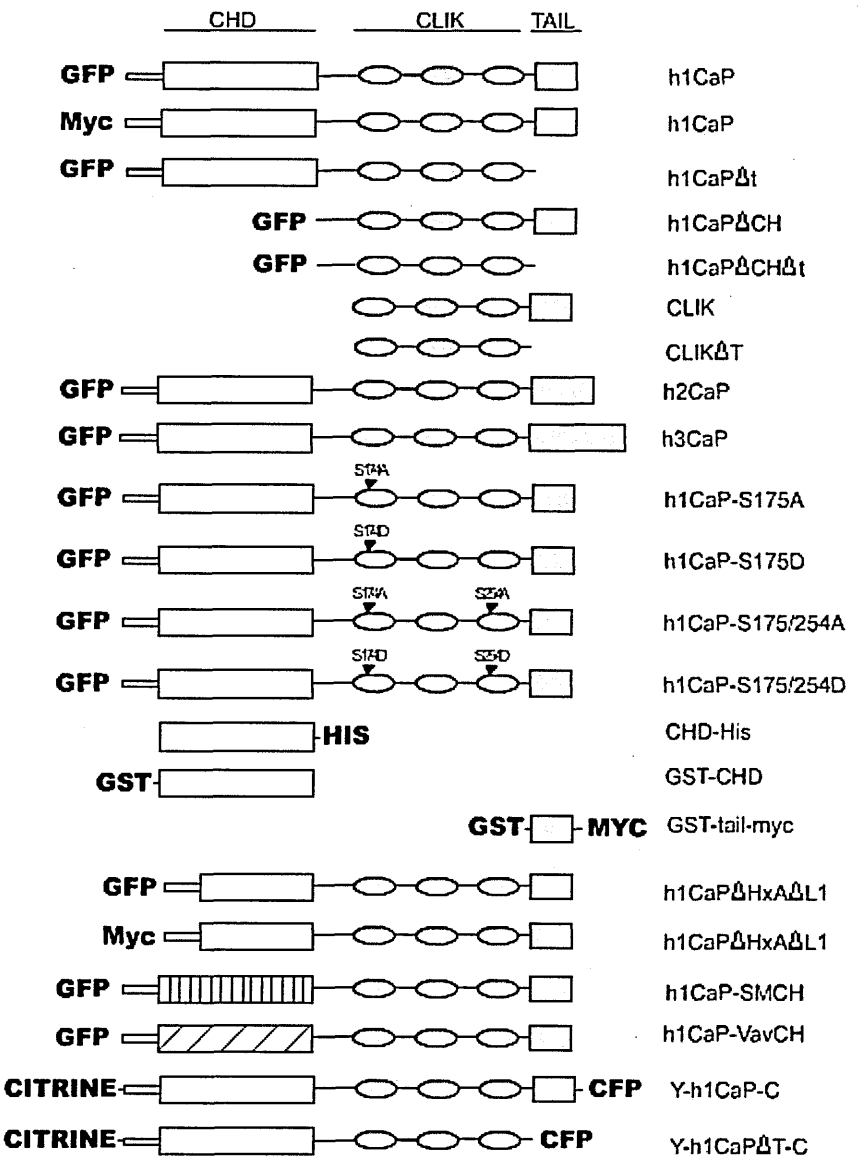


Figure 10. CaP constructs used in this study. The main characteristic domains of the CaPs were kept aligned to facilitate the reading.

2.4 Computational docking

The nucleotide sequence of Mus musculus h1CaP C-terminal tail was obtained from the data-bank in the National Center for Biotechnology Information (*http://www.ncbi.nlm.nih.gov/*). The crystal structure of the rabbit muscle creatine kinase (PDB code, 2CRK) was downloaded from Protein Data Bank. The alignment between the C-terminal tail of h1CaP and the creatine kinase was obtained using the web-server NCBI blastp (*http://blast.ncbi.nlm.nih.gov/*). The computational docking models were generated with the FTDOCK algorithm, which permits an exhaustive search of translational and rotational space (Gabb et. al. 1997). The modelling of the tail 3D structure was determined by a segmented approach where the C-terminal tail of h1CaP was isolated and modelled with an individual template. A 33% homologueous sequence of the rabbit creatine kinase (EYPDLSKHNNH) was used as a template for the tail (LNPEYPELSEPTHNHHPHNYYNSA). Swiss PDB viewer was used to model the tail. The quality of the model was evaluated using the HOPPscore application (Sims and Kim, 2006) and with Ramachandran plot from VMD molecular graphic programme (Humphrey et al., 1996). Negative FTDOCK scores indicate overlap/interpenetration of the ligand and receptor and are therefore not possible. A score of zero indicates that the ligand and receptor do not interact at all, and large positive scores denote complex formation with good surface complementarity. The addition of the molecular surface volumes were performed in AutoDockTools 1.5.1 (Sanner, 1999). The surface electrostatic potentials, the solvation energy and the binding energy were assigned and analyzed with the adaptive Poisson-Boltzman solver (ABPS), which is included in AutoDockTools as a subroutine (Baker et al., 2001).

2.5 Protein purification

The recombinant CH domain of h1CaP spanning the residues D27 to K134 was generated with the glutathione-S-transferase (GST) or 6xHis fusion protein system. The C-terminal tail of h1CaP spanning the residues L271 to A297 was generated with a C-terminal GST tag and an N-terminal myc tag. The bacteria strain BL21(DE3) was transformed with the expression vectors encoding for the recombinant proteins and grown to an optical density at 600 nm (OD_{600}) of 0.4-0.6 at 37 °C. Expression of the protein was induced with the addition of isopropyl thio-β-D-galactoside (IPTG, Calbiochem) and incubating the cells for 4 h at 30 °C. Subsequently, cells were harvested by centrifugation at 6000x *g* and stored at –80 °C overnight. The cells with GST fused proteins were resuspended in 20 mM Tris, 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma), and lysed mechanically with a sonicator (Vibra Cell, SONICS). The lysates were centrifuged at 15,000x *g* for 15 min, and the pellet containing the cell debris was discarded. The supernatant was mixed with glutathatione sepharose beads (Amersham) for 3 h at 4 °C on a rotating support. Beads were packed into a 10 ml chromatography column (Bio-Rad) to purify the recombinant GST fused proteins. The beads were washed twice with 10 ml of PBS (pH 7.4) and eluted with 50 mM Tris (pH 8), 10 mM reduced glutathione and 1 mM PMSF. The eluates were dialysed against 20 mM Tris and 5 mM EDTA. Protein concentration was determined by the Bradford method using Bio-Rad protein-assay reagent. The purity of the eluted proteins was analyzed by SDS-PAGE and staining the gels with Coomassie blue.

Cells with His fused proteins were resuspended in 20 mM $NaHPO_4$ (pH 7.4), 0.5 M NaCl and 1 mM PMSF, and mechanically lysed with a sonicator. The lysates were centrifuged at 15,000x *g* for 15 min, and the pellets containing the cell debris

were discarded. The supernatant was applied to 1 ml Ni-conjugated-hiTrap Chelating HP column (Amersham) following the manufacturer protocol. The column was washed with 5 volumes of 0.02M $NaHPO_4$, 1M NaCl and 10mM imidazole (pH 7.2) and proteins were eluted with 0.02 M $NaHPO_4$, 0.5 M NaCl and a 0.05 to 0.5 M imidazole gradient (pH 7.4). The fraction containing the His fused proteins was determined by measuring the absorbance of the eluates at OD_{280}. The eluates containing the His fusion proteins were dialysed against 20 mM Tris and 5 mM EDTA. Protein concentration was determined by Bradford method using Bio-Rad protein-assay reagent. The purity of the proteins was analyzed by SDS-PAGE and staining the gels with Coomassie blue.

2.6 Overlay assay

Pure GST-tail-myc, GST-CHD or CHD-His were spotted onto a nitrocellulose membrane. The blots were blocked with 20 mM Tris, 136 mM NaCl, 0.05% Tween20 (TBS-T) with 5% milk for 1 h at room temperature. Blots with immobilized GST-CHD or CHD-His were incubated with TBS-T containing 10µg of GST-tail-myc for 2 h at room temperature whilst blots with immobilized GST-tail-myc were incubated for the same period of time with TBS-T containing 10µg of GST-CHD or CHD-His. After incubation the blots were rinsed once with TBS-T and PBS was immediately added with 4% PFA for 1 min. Then blots were washed three times with TBS-T and proceeded with the detection with the corresponding antibodies, as for Western blotting.

2.7 Fluorescence resonance energy transfer (FRET)

FRET assays were performed with transiently transfected A7r5 cells plated in a 30 mm glass-bottomed dishes (Matek) for live-cell imaging. Before imaging, 25 mM HEPES was added to the media. Images were recorded at 37 °C on a Zeiss LSM510 META confocal microscope equipped with a 63x, 1.4NA Plan-APOCHROMAT objective (Zeiss). CFP was excited with a 458nm Ar-ion laser. Emissions of Y-h1CaP-C and Y-h1CaPΔT-C were collected with the Meta detector to analyze the spectral emission of each construct. FRET was measured using the donor dequenching technique (Bastiaens and Jovin, 1996), in which the fluorescence of the CFP is monitored after bleaching the acceptor (YFP). Two images were recorded before bleaching and a third image immediately after bleaching the YFP in a region of interest (ROI) within the cell. The outer region of the ROI was used as a control. The values of the fluorescence intensities were recorded before (IbB) and after bleaching (IaB) in the ROI and outside the ROI. The differences between IaB – IbB were considered as the variations in the fluorescence intensities of the CFP. The variation of the fluorescence outside the ROI before and after bleaching was relatively low; therefore, it was not necessary to apply a correction due to laser power fluctuations or for the exposure of the cell to the light of the laser during bleaching. A student's T-test was applied to analyze if the variations between the samples are significant.

2.8 Fluorescence recovery after photobleaching (FRAP)

FRAP was performed in transiently transfected A7r5 cells with GFP fused h1CaP, h1CaPΔT, h1CaPΔCHD and h1CaPΔCHDΔT. The cells were plated in a 30 mm glass-bottomed dishes and monitored at 37 °C on a Zeiss LSM510 META confocal microscope equipped with a 63x, 1.4NA Plan-APOCHROMAT objective

(Zeiss). Two images were recorded before bleaching, a third image immediately after bleaching a region of interest (ROI) within the cell containing stress fibres, and a fourth image 2 or 5 minutes after bleaching. The outer region of the ROI (OROI) was used as a control. The values of the fluorescence intensities were recorded in the ROI and OROI before (IbB) and after 2 or 5 minutes of bleaching (IaB). Variation in the fluorescence of GFP in the non-bleached area can occur due to laser-power fluctuations, natural bleaching of the fluorophores or loss of the focal plane during the recording time. Hence, the ratio between IbB_{OROI}/IaB_{OROI} in the OROI was used as the correction value for the IaB of the ROI (IaB_{corr}):

$$IaB_{corr} = (IbB_{OROI}/IaB_{OROI})*IaB_{ROI}$$

The IaB_{corr} was then used to obtain the percentage of fluorescence recovery, considering IbB_{ROI} as 100% of fluorescence.

CHAPTER 3: RESULTS I

H1CaP has been demonstrated to be an ABP that stabilizes actin filaments and regulates smooth muscle contraction. However it is still unclear how the actin-binding activity of h1CaP is regulated and no regulatory mechanism has been described in detail. One of the first regulatory mechanisms to be proposed was the binding of Ca^{2+}/CaM (Abe et al., 1990; Mezgueldi et al., 1992) to h1CaP, which inhibits the interaction with actin and the h1CaP-mediated inhibition of the MgATPase activity of myosin II. Nevertheless, *in-vivo* experiments are needed to corroborate if such mechanism occurs in cells. Another regulatory mechanism could be mediated by h1CaP phosphorylation. It has been shown that PKC and ERK1/2 bind h1CaP, but the biological significance of these interactions remains elusive. Phosphorylation of h1CaP significantly reduces its actin-binding activity and suppresses its ability to inhibit the actin-activated ATPase activity of myosin. It has been shown that h1CaP can be phosphorylated *in vitro* by PKC , Ca^{2+}/CaM-dependent protein kinase II (Ca^{2+}/CaMKII), ROCK (Kaneko et al., 2000; Winder and Walsh, 1990a; Winder and Walsh, 1990b) and Fyn (Abouzaglou et al., 2004). The main phosphorylated sites produced by PKC, Ca^{2+}/CaMKII and ROCK include Ser175 and Ser254, while Fyn phosphorylates mainly Tyr182 and Tyr261. However it is still unknown whether phosphorylation of h1CaP occurs *in vivo* or not. While some laboratories have detected phosphorylated h1CaP upon muscle contraction after incubation with radio labelled isotopes (Carmichael et al., 1994; Gerthoffer and Pohl, 1994; Mino et al., 1995; Winder et al., 1993a), other groups have failed to do so (Adam et al., 1995; Barany and Barany, 1993; Barany et al., 1991; Gimona et al., 1992). In any case, phosphorylation of Ser175 of h1CaP is sufficient to inhibit its interaction with actin *in*

vitro (Tang et al., 1996; Uyama et al., 1996), but *in vivo* evidence for regulation by Ser/Thr phosphorylation is missing.

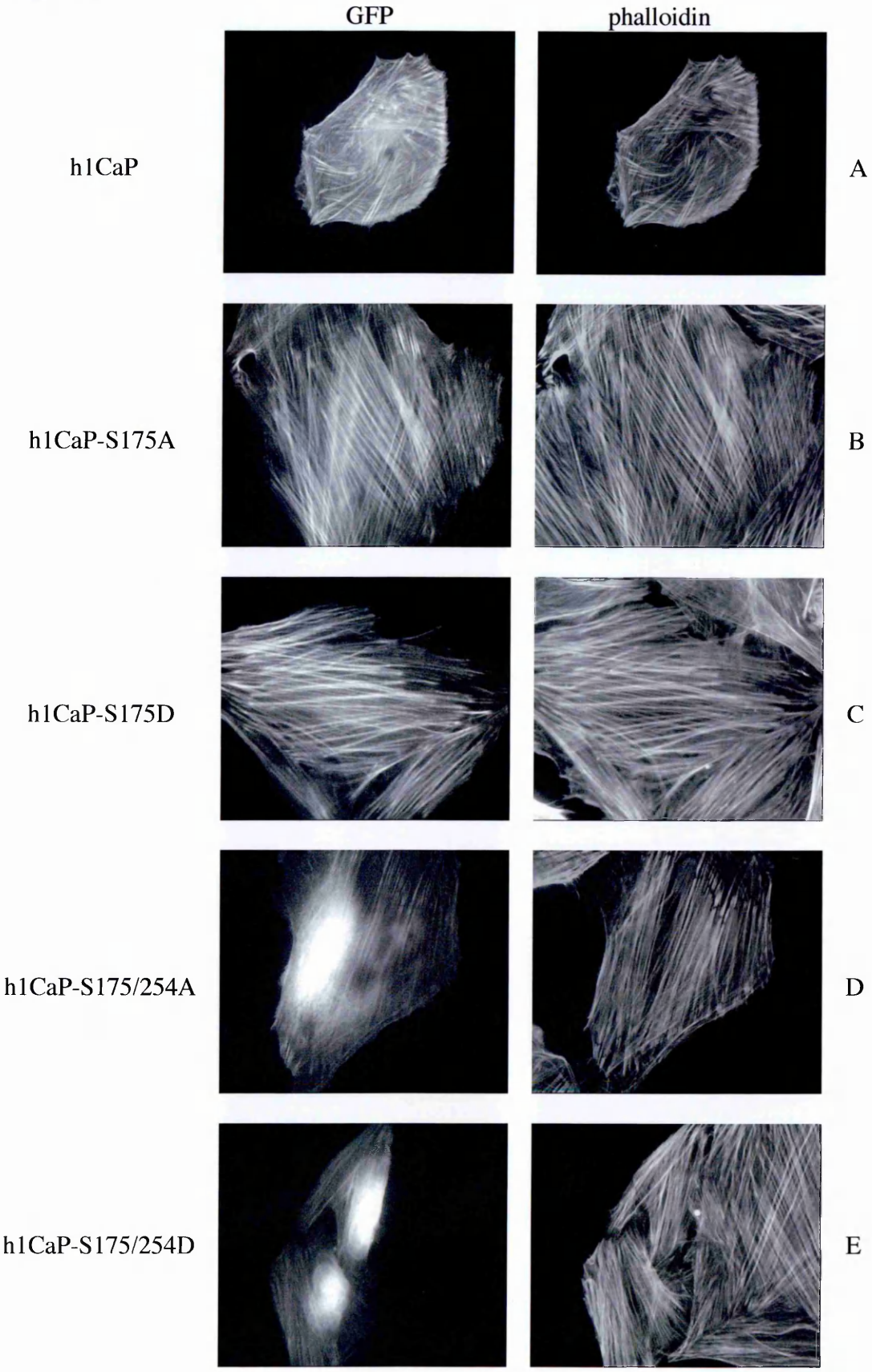
3.1 Phosphorylation may not regulate the actin-binding activity of h1CaP

GFP tagged h1CaP decorates and colocalizes with actin filaments. Therefore, to understand whether phosphorylation regulates the actin-binding activity of h1CaP in live cells, I have compared the effects on actin colocalization of h1CaP site-specific mutants in which Ser175 was replaced by alanine (h1CaP-Ser175A) or aspartate (h1CaP-Ser175D). To analyze the effects of both Ser175 and Ser254, I also compared the effects of a double mutant in which Ser175 and Ser254 had been replaced for either alanine (h1CaP-Ser175/254A) or aspartate (h1CaP-Ser175/254D). The reason for exchanging the serine for an aspartate was to mimic the phosphorylated form of h1CaP while the presence of an alanine renders the residue non-phosphorylatable. The constructs were transfected into A7r5 rat smooth muscle cells, which contain a high density of actin filaments that allows the study of actin-binding proteins and, also express endogenous h1CaP. Figure 11 shows that h1CaP-S175A and h1CaP-S175D colocalize with actin filaments similar to wild-type h1CaP (Fig. 11A, B and C). This suggests that phosphorylation of Ser175 does not affect the actin-binding activity of h1CaP in resting interphase cells. This result goes contrary to earlier results, where it was shown that the exchange of Ser175 for an alanine or aspartate is sufficient to inhibit h1CaP actin binding *in vitro* (Tang et al., 1996; Uyama et al., 1996). However, it cannot be ruled out that the association of h1CaP-S175A and h1CaP-S175D with actin filaments in cells could be indirectly and mediated by binding tropomyosin (Takahashi et al., 1988) or caldesmon (Graceffa et al., 1996).

On the other hand, cells overexpressing h1CaP-Ser175/254A and h1CaP-Ser175/254D show low levels of colocalization of the GFP-tagged proteins with actin filaments and the proteins are mainly found in the cytoplasm and the nucleus, regardless of the type of mutation (Fig. 11D and E). The fact that the double-mutation constructs showed similar defects, this does not allow determination of whether the low levels of colocalization are due to the negative charges of the aspartates that mimic phosphorylation or to a critical role of both serines in mediating h1CaP actin binding. Another aspect that has not been addressed yet in live cells regards the tyrosine phosphorylation of h1CaP. Sodium orthovanadate (Na_3VO_4) is a broad-spectrum inhibitor of tyrosine phosphatases. It binds to and blocks the active sites of phosphatases, and hence phosphatases can no longer dephosphorylate their target proteins. Therefore, I transfected A7r5 cells with GFP-tagged h1CaP and incubated these cells with Na_3VO_4 for 30 minutes to block the activity of tyrosine phosphatases; no differences between treated and non-treated cells was detected (data not shown). These results further suggest that tyrosine phosphorylation is not the main regulatory mechanism of the actin-binding activity of h1CaP in live cells.

Figure 11. (page 70) Subcellular localization of h1CaP constructs tagged with GFP in A7r5 cells. (A) H1CaP is observed on stress fibres and a less intense signal of GFP is observed in the cytoplasm. (B) Mutation of Ser175 for an alanine or (C) aspartate did not affect the localization of h1CaP. (D) Mutation of the Ser175 and Ser 254 for an alanine or (E) aspartate significantly affected the cellular localization of h1CaP.

Figure 11


GFP
phalloidin
h1CaP
A
h1CaP-S175A
B
h1CaP-S175D
C
h1CaP-S175/254A
D
h1CaP-S175/254D
E

3.2 The CH domain of h1CaP is required but not sufficient for actin binding

Unlike the type 1 CH domains, the biological role of the type 2 and 3 CH domains is still unclear since they both lack actin-binding activity. However, it is assumed that the type 2 CH domains cooperate with type 1 CH domains to bind actin filaments (Lorenzi and Gimona, 2008). It was shown before that the CH domain of h1CaP does not bind to actin filaments and to date there is no clear concept for its biological function (Gimona and Mital, 1998). While the CLIK^{23} repeats directly interact with actin filaments (Galkin et al., 2006) and the C-terminal tail of h1CaP negatively regulates the actin-binding activity of h1CaP (Burgstaller et al., 2002) there is no clear evidence for a potential contribution of the CH domain to the actin-binding activity of h1CaP. Therefore in an attempt to gain more information about the contribution and the behaviour of the CH domain and the C-terminal tail of h1CaP on the actin-binding activity in cells, I have used GFP- labelled h1CaP probes to study their localization within the cell. A7r5 cells were transfected with: 1) full length h1CaP (h1CaP); 2) h1CaP without the tail (h1CaPΔT); 3) h1CaP without the CH domain (h1CaPΔCH); and 4) h1CaP without the CH domain and the tail (h1CaPΔCHΔT). The cells were fixed and observed under an upright fluorescence microscope, and phalloidin staining was performed to visualize F-actin. Figure 12 shows minor variations in stress fibre localization between h1CaP and h1CaPΔT (Fig. 12A and B). H1CaP is mainly associated with stress fibres and a weak fluorescent signal is observed in the cytoplasm. In contrast, h1CaPΔT is almost exclusively observed on stress fibres. These data are in agreement with previous results that have demonstrated that h1CaP lacking its C-terminal tail is more strongly retained in an insoluble cytoskeletal fraction (Burgstaller et al., 2002; Danninger and Gimona, 2000). On the other hand, h1CaPΔCH failed to prominently decorate actin stress

fibres (Fig. 12C), and a stronger GFP signal is observed in the cytoplasm, suggesting that h1CaP without the CH domain binds poorly to actin filaments. This suggests that the CH domain influences the interaction of h1CaP with actin filaments; however, it was shown previously that the CH domain of h1CaP does not bind actin filaments directly (Gimona and Mital, 1998). Therefore, it is possible that the CH domain of h1CaP plays a regulatory role on the actin-binding activity of h1CaP. Interestingly, the localization of h1CaPΔCHΔT is similar to that observed for full-length h1CaP (Fig12A and D). This h1CaPΔCHΔT comprises mainly the CLIK^{23} repeats, which is the domain responsible for the actin-binding activity of h1CaP.

These results, together with previous biochemical studies on h1CaP (Bartegi et al., 1999; Burgstaller et al., 2002; Danninger and Gimona, 2000; Galkin et al., 2006; Gimona and Mital, 1998) allow new hypotheses to be explored as to how the actin-binding activity of h1CaP is regulated. Figure 12C and 12D demonstrate a possible scenario in which the absence of the CH domain favours an interference of the C-terminal tail of h1CaP with the actin-binding region. Therefore, one can assume that the documented negative effect of the C-terminal tail on the actin-binding activity of h1CaP is blocked by the presence of the N-terminal CH domain. Furthermore, intrinsic regulatory mechanism may explain the observation of two h1CaP conformations (Stafford et al., 1995). One conformation could be mediated by the interaction between the CH domain and the C-terminal tail, allowing CLIK^{23} repeat-mediated actin binding. The second conformation could be characterized by the interaction between the acidic tail with the positively charged residues on the CLIK^{23} repeats which inhibits the actin-binding activity of h1CaP.

To test this hypothesis of a potential influence of the C-terminal tail on the actin-binding activity of the CLIK^{23} repeats I generated two constructs coding for the

CLIK^{23} repeats with (CLIK) or without the C-terminal tail (CLIKΔT) tagged with GFP. The constructs were transfected in A7r5 cells to analyze their subcellular localization. Figure 13 shows that the CLIK^{23} repeats without the tail are able to decorate actin filaments (Fig. 13B), whereas the CLIK^{23} repeats with the tail fail to do so (Fig. 13C). This result suggests that the tail exerts its inhibitory effect by interfering with the actin-binding activity of the CLIK^{23} repeats.

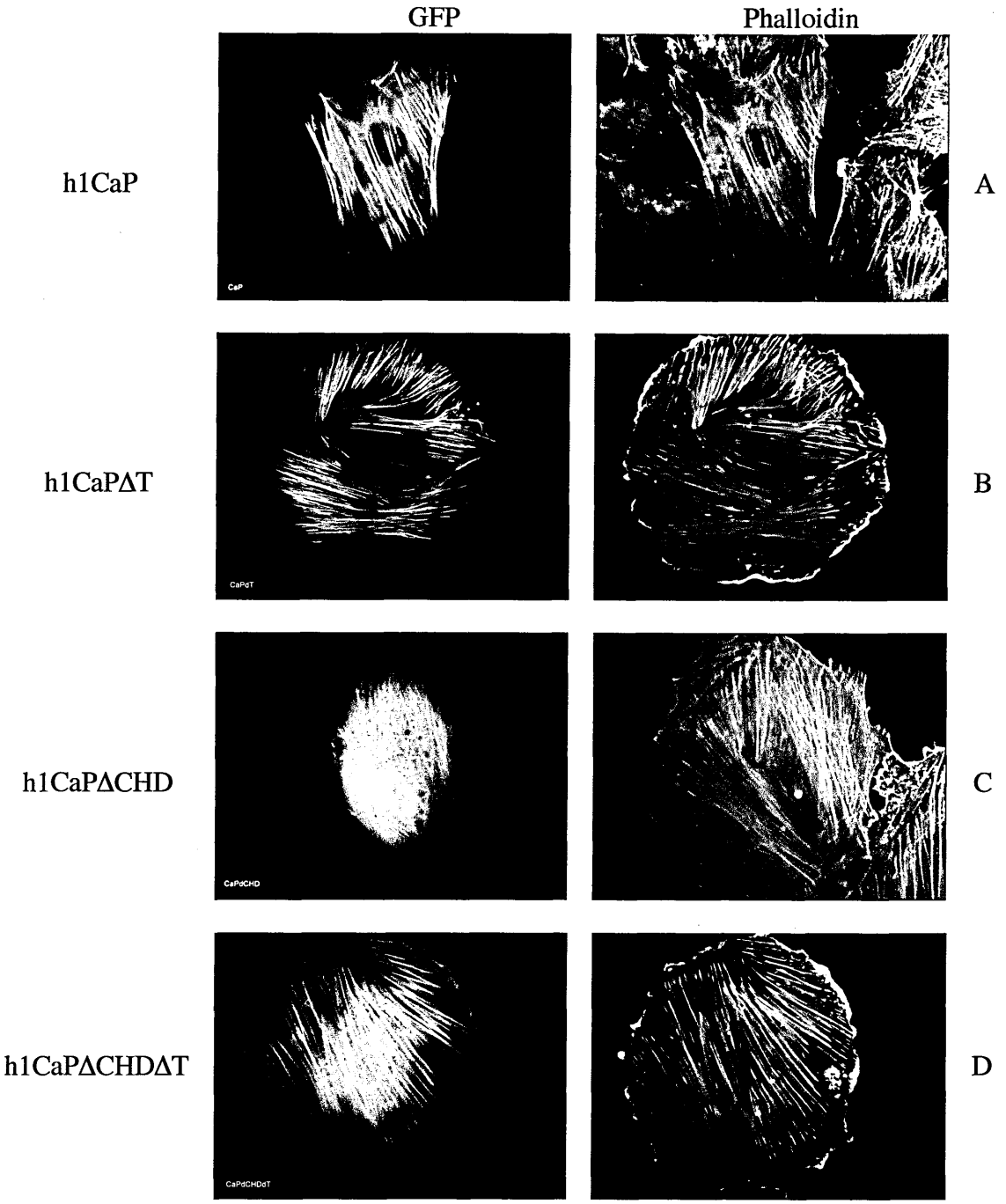


Figure 12. Subcellular localization of h1CaP constructs. (A) H1CaP is observed mainly on stress fibres and partially in the cytoplasm (see also Fig. 1A). (B) The tail-deleted form of h1CaP is only found on stress fibres. (C) Most of the CH domain-deleted form of h1CaP is observed in the cytoplasm. (D) H1CaP lacking both the CH domain and the tail localizes mainly with stress fibres and there is little in the cytoplasm.

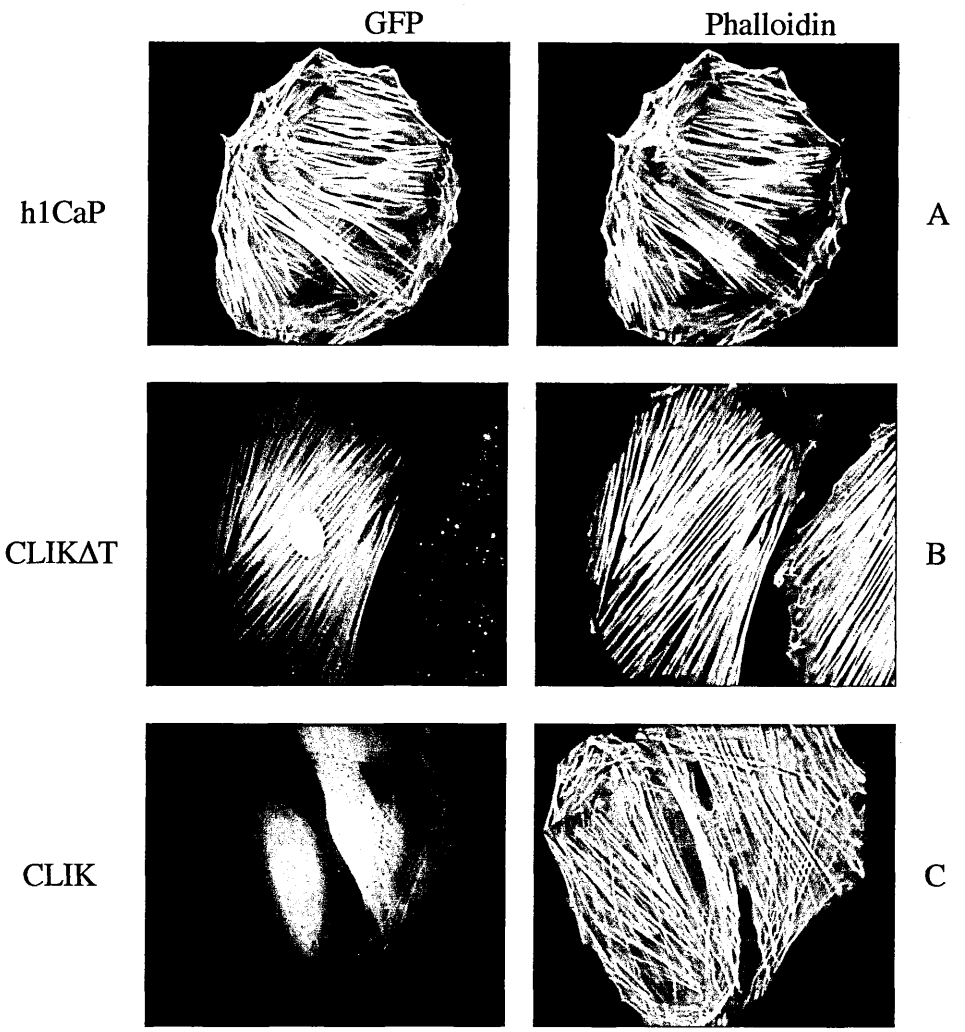


Figure 13. Subcellular localization of h1CaP and the isolated CLIK[23] repeat module tagged with GFP. (A) Most of H1CaP localizes with stress fibres (See also Fig. 1A and 2A). (B) The CLIK[23] repeats lacking the C-terminal tail are found mainly on stress fibres and to some extent inside the nucleus. (C) The CLIK[23] repeats carrying the tail localize mainly in the cytoplasm of the cells as well as in the nucleus.

3.3 *In silico* interaction of the CH domain and the tail of h1CaP

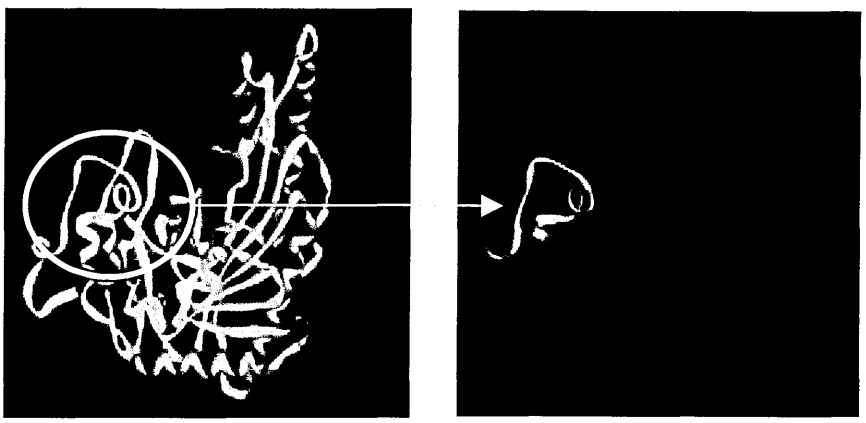
As a first approach to examine whether the CH domain and the tail of h1CaP interact, a molecular docking algorithm (FTDOCK) was used (Gabb et al., 1997; Katchalski-Katzir et al., 1992). This programme requires the crystal or solution structure of the two molecules that are to be docked. While the solution structure of the CH domain of h1CaP was solved a few years ago (Bramham et al., 2002), the structure of the rest of the protein remains elusive. Thus the shape of the C-terminal tail of h1CaP was *modelled* by sequence homology. The amino-acid sequence of the tail was blasted (NCBI Blastp), and a portion of the sequence of the creatine kinase protein showed 33% homology at the amino-acid level (Fig. 14).

```
LNPEYPELSEPTHNHHPHNYYNSA   Tail of CaPh1 (L274 to A297)
   :::+::    :: :
SEEEYPDLS--KHNNHMAKVLTPD   Creatine kinase sequence (S16 to D37)
```

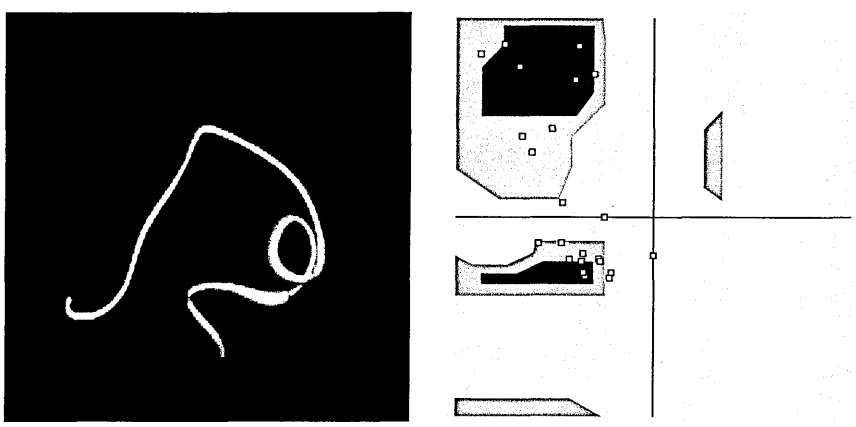
Figure 14. Comparison of the amino-acid sequences of the C-terminal tail of h1CaP and the homologous sequence of creatine kinase. (+ similar charges)

Therefore the known crystal structure of this portion of creatine kinase was used as template to model the shape of the h1CaP tail (Fig. 15). The quality of the tail structure was evaluated with the HOPPscore application (Sims and Kim, 2006) and with the Ramachandran plot from the VMD molecular graphic programme (Humphrey et al., 1996).

FTDOCK requires a static molecule to fit a ligand. Thus, the CH domain was assigned as static and the tail was used as a free molecule to be positioned around the CH domain. The same analysis was performed with a scrambled tail sequence



Creatine kinase crystal structure homologous sequence in h1CaP



CaPh1 tail after modelling Ramachandran plot

Figure 15. Homology modelling of the C-terminal tail of h1CaP. The known structure of creatine kinase was used as template to model the shape of the tail. The final structure was evaluated with the HOPPscore application and with the Ramachandran plot.

(tailScmbl) and with the creatine kinase sequence as, controls. The tailScmbl was generated by randomly mixing the residues of the h1CaP tail, while for the second control, the sequence of creatine kinase corresponding to the homology sequence of the tail was used.

Unlike the two controls, the tail was positioned predominantly facing the helix A and loop 1 of the CH domain (Fig. 16A), with a large contact area, and six out of the 10 best results were positioned at almost identical positions. On the other hand, no contact area was seen between the scrambled tail and the CH domain, and the tail was oriented away from the CH domain, with a prediction for interaction with just one single residue (Fig. 16B). In addition, the docking of the creatine kinase sequence onto the CH domain did not show any preferential position (Fig. 16C).

To extend the above analysis, the primary regions and residues predicted to be involved in the interaction between the h1CaP CH domain and tail were identified (Fig. 17). The molecular surface was computed with AutoDockTools 1.5.1 (Sanner, 1999) and the electrostatic surface potentials with the APBS algorithm (Baker et al., 2001). When the surface volume and charges are assigned to the docked parts of the CaP molecule, both the surface profiles and charge oppositions matched. The tail showed a cleft into which the Arg44 of the CH domain can be inserted. Arg45 and Asn49, which are also positioned on helix A/loop 1 of the CH domain, might contribute to the stabilization of this interaction.

Electrostatic interactions play an important role in determining the thermodynamics of binding and they can influence long-range attractions between the substrate and the ligand (Gabdoulline and Wade, 2002). The favourable charge – charge interactions between the ligand and the receptor help in the formation of the complex and stabilizing low energy interactions such as salt bridges and hydrogen

bonds (Chong et al., 1998; Schreiber and Fersht, 1995). However, the affinity between the ligand and the receptor molecule must be strong enough to shed their bound water molecules in order to allow close binding. Therefore, the solvation and binding energies of the complex resulted from the FTDOCK were calculated. The results are summarized in Table 3. Fastcontact 2.0 (Camacho and Zhang, 2005) was used to analyze the major residues that may contribute to the electrostatic energy (4r) between the CH domain and the tail. The best three residues of the CH domain identified using Fastcontact were Arg44, Arg45 and Asn49 with 4r of -20.54, -19.04 and -20.78 kJ/mol respectively. On the other hand, the best three residues of the tail were Glu280, Asn292 and Tyr293 with 4r of -24.65, -10.07 and -9.94 kJ/mol, respectively. In addition, with Fastcontact it was possible to identified the major receptor-ligand residue electrostatic contacts, which included Asn49 – Glu280, Arg44 – Glu283 and Arg45 – Asn292, with 4r of -16.34, -11.57 and -11.57 kJ/mol, respectively. Although the electrostatic energies at the single residue level are high, the calculated binding energy of the complex formed by the tail and CH domain region comprising the helix A and the loop 1 is relatively low (table 3). Indeed, immunoglobulin binding energies range from -20 kJ/mol to -130 kJ/mol (Schwarz et al., 1995). However, low binding energies might account for a more dynamic interaction.

	ΔG solvation (kJ/mol)	Binding energy (kJ/mol)
tail	-2814.52	-
CH domain	-2922.87	-
complex	-5363.59	-5.36

Table 3. Solvation and binding energies calculated with the APBS algorithm (Baker et al., 2001). Only the region comprising the helix A and the loop 1 was used for the energy calculations of the CH domain. Energies were calculated considering the solvent dielectric of water (78.54) at 298.15 K (25 °C).

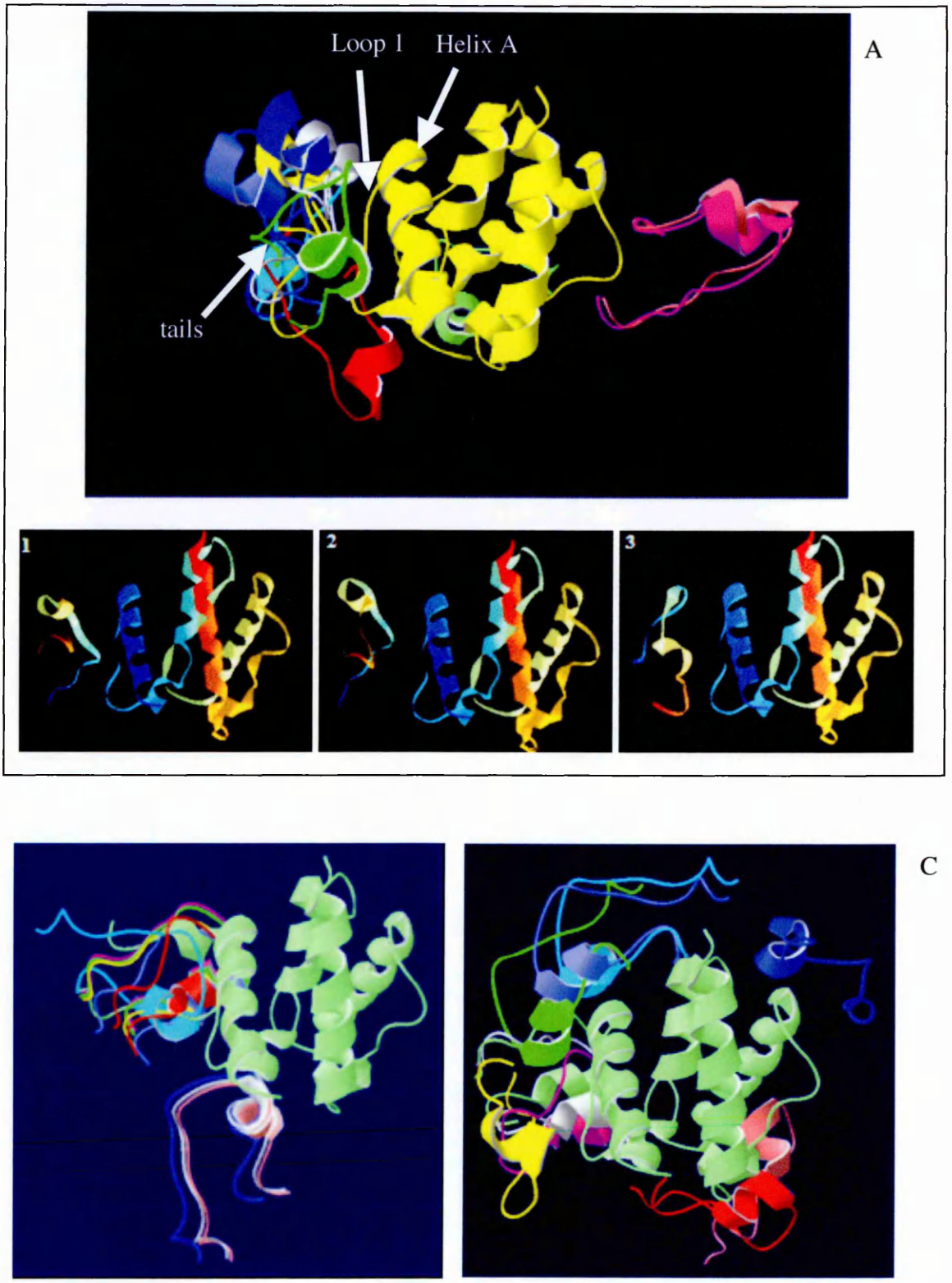
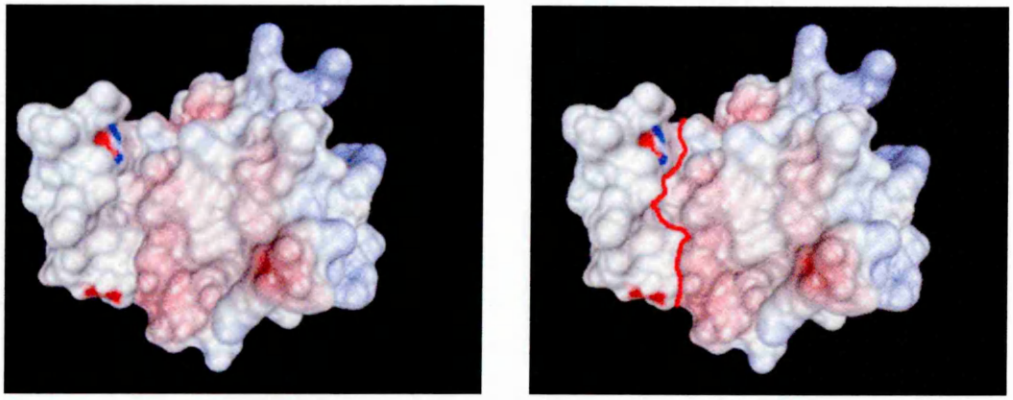


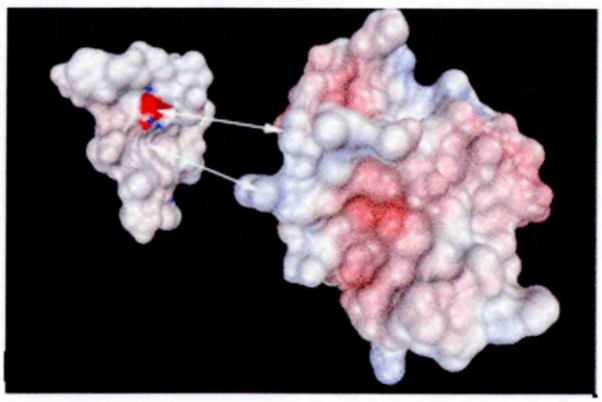
Figure 16. Docking the C-terminal tail onto the CH domain of h1CaP. (A) The tail is predicted to contact with the helix A and loop 1 of the CH domain. The best three docking results are in similar positions. (B) No contact area is seen between the scrambled tail and the CH domain, and the tail is oriented away from the CH domain interacting with just one residue. (C) The docking of the creatine kinase sequence onto the CH domain does not show any predominant position.

A

B



C



D

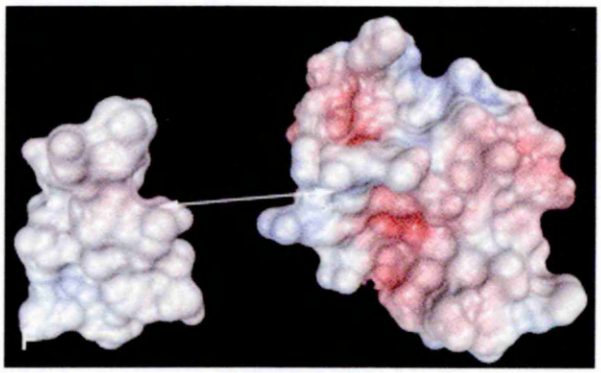
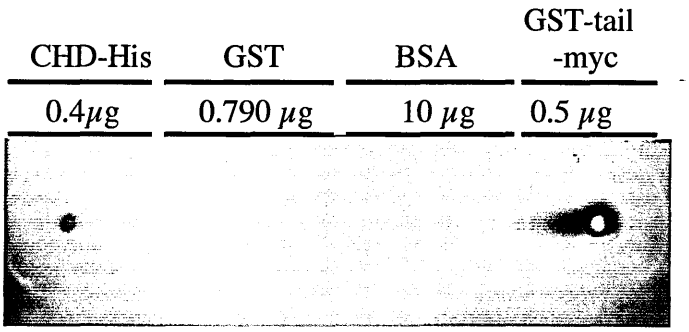


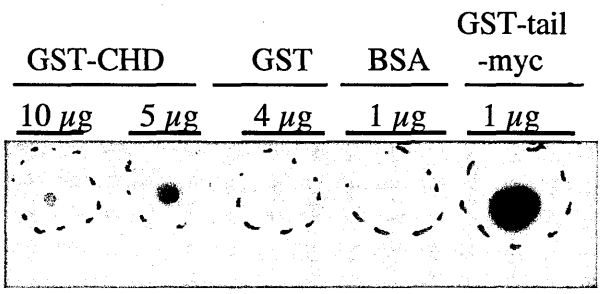
Figure 17. Analysis of the regions involved in the interaction between the CH domain and the tail of h1CaP. The surface electrostatic potentials were mapped in MGT tools using APBS (Baker et al., 2001) (A) Surface with charges of the complex formed by the tail and the CH domain. Positive charges are represented in blue and negative charges in red shadings. (B) A red line marks the limits between both domains. (C) The CH domain and the tail were separated and rotated in order to observe the residues involved in the interaction. The arrows mark the Arg44 and Arg45 of the CH domain. The CH domain is inserted into the cleft of the tail. (D) A second area of contact comprising Asn49 of the CH domain is revealed when the complex is viewed from a different angle.

3.4 The h1CaP CH domain and the tail interact *in vitro*

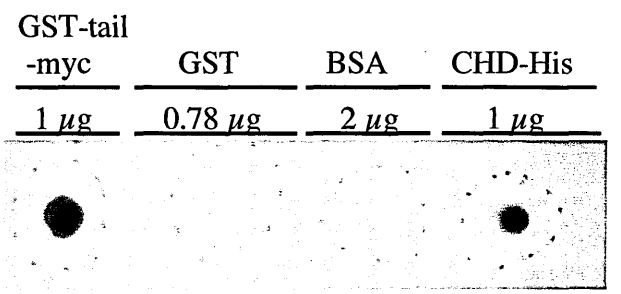
To further explore the significance of the intramolecular interaction between the h1CaP CH domain and the tail, both domains were used to perform an *in-vitro* overlay assay. His-tagged (CHD-His) or GST-tagged (GST-CHD) CH domains, and the tail tagged with GST and myc (GST-tail-myc) were expressed in bacteria and purified. Either the CH domain or the tail were immobilized on a nitrocellulose membrane and incubated with a solution containing the ligand. Specific antibodies against the tags present on the proteins in solution (ligand) where used to detect binding. When GST-tail-myc was immobilized on the nitrocellulose membrane the CHD-His was detected bound to the tail and no signal was detected in the controls (Fig. 18C). Similar results were obtained using the GST-CHD or CHD-His as the immobilized molecule (Fig. 18A and B). In both cases the tail was detected bound to the CH domain. This data strongly suggests that the tail and the CH domain interact directly. However, the requirement for the addition of paraformaldehyde to maintain the interaction suggests that the interaction between the h1CaP CH domain and the tail is weak.



A



B



C

Figure 18. *In-vitro* interaction between the h1CaP CH domain and tail. (A) The CH domain tagged with His for detection was immobilized on a nitrocellulose membrane and incubated with a solution containing the tail tagged with GST and myc. After several washes the tail was detected with an anti-myc antibody. (B) The tail of h1CaP interacts with a GST-tagged CH domain. (C) The tail tagged with a GST and myc was immobilized on a nitrocellulose membrane and incubated with a solution containing the CH domain tagged with His. After several washes the CH domain was detected using an anti-His antibody.

3.5 Helix A and the loop 1 of the h1CaP CH domain are involved in the regulation of its actin-binding function

The results obtained from the *in-silico* docking studies between the h1CaP CH domain and tail suggested that helix A and loop 1 of the CH domain are involved in mediating this interaction. Therefore, a recombinant h1CaP lacking the potential region of interaction in the CH domain should show reduced actin-binding activity. To examine whether the helix A and loop 1 of the CH domain of h1CaP play a role in the actin-binding function of h1CaP I generated two constructs where the helix A and the loop 1 of the CH domain were deleted (h1CaPΔHxAΔL1) and tagged with either myc or GFP for detection. Deletion of helix A and loop 1 of the CH domain significantly reduced the actin-binding activity of h1CaP (Fig. 19 and 20). This result is in agreement with the underlying hypothesis that the h1CaP CH domain modulates the inhibitory role of the h1CaP C-terminal tail.

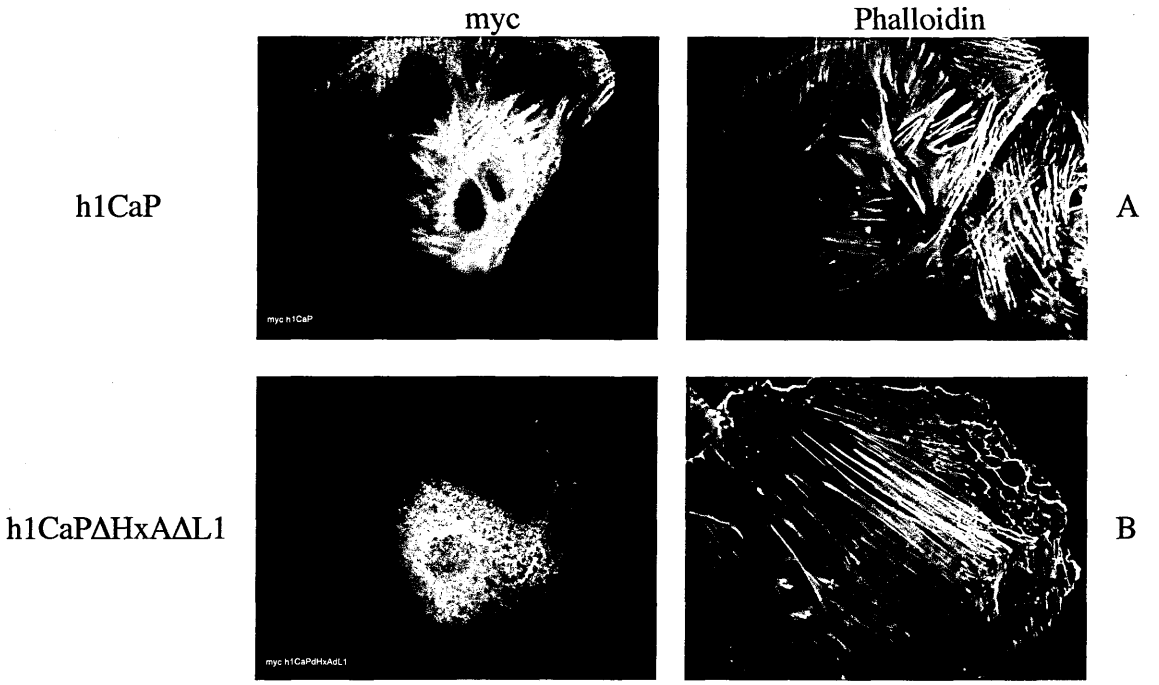


Figure 19. Subcellular localization of myc-tagged h1CaP constructs in A7r5 cells. (A) H1CaP localizes preferentially with stress fibres and little is found in the cytoplasm. (B) H1CaP lacking the helix A and the loop 1 of the CH domain is mainly found in the cytoplasm.

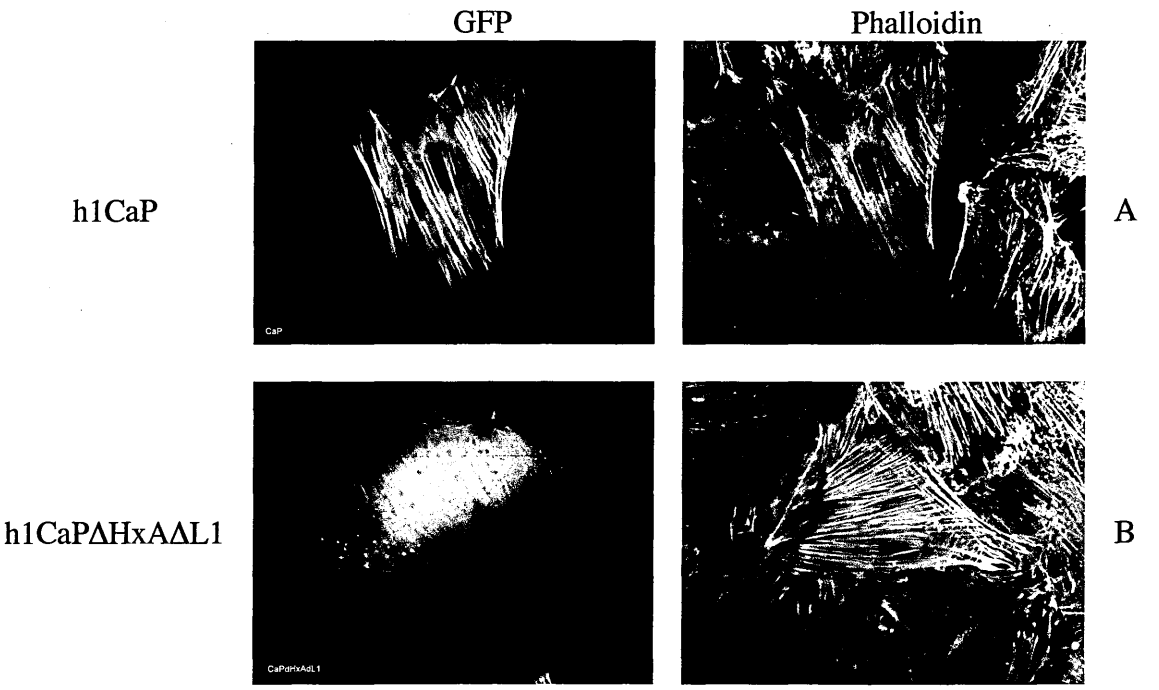


Figure 20. Subcellular localization of GFP-tagged h1CaP constructs. (A) H1CaP localizes preferentially with stress fibres and little is found in the cytoplasm of the cell. (B) H1CaP lacking the helix A and the loop 1 of the CH domain is mainly found in the cytoplasm.

3.6 The CH domains of CaP, SM22 and Vav are not interchangeable

Although CH domains from different proteins have low sequence similarities, a few defined residues are almost always invariant. These core residues are thought to be the major factors involved in establishing the three dimensional structure of the CH domain (Gimona et al., 2002), and the differences observed in the sequences explain the functional diversity observed among the CH domains. H1CaP, Vav and SM22α have in common a type 3 CH domain (Fig. 21). The CH domain of Vav has been found to regulate the transforming activity of the protein through an intramolecular interaction with the zinc finger (ZF) and Dbl homology (DH) domains (Zugaza et al., 2002), and a similar regulatory mechanism has been reported for ARHGEF6 (Daniels et al., 1999). Therefore, type 3 CH domains might serve a general intramolecular regulatory function.

Sequence comparisons between the CH domains of SM22, Vav and h1CaP show differences in the helix A and loop 1 (Fig. 21). The predicted residues involved in the interaction between the h1CaP CH domain and the tail are missing in both, SM22 and Vav. Thus, to extend the analysis, the CH domain of h1CaP was replaced by the CH domain of SM22 and Vav in order to investigate whether the regulatory function that was identified in the CH domain of h1CaP is conserved in other type 3 CH domains. H1CaP carrying the CH domains of SM22α (h1CaP-SMCH) or Vav (h1CaP-VavCH) were generated as GFP fusion cDNA constructs and cells were transfected with these probes. Phalloidin was used to visualize F-actin.

Figure 22 shows that neither the CH domain of Vav nor that of SM22α is able to substitute for the CH domain of h1CaP. H1CaP-SMCHD poorly decorates actin filaments and a stronger cyoplasmic fluorescent signal is observed (Fig. 12A and C); its localization in the cell resembles more the predominantly cytoplasmic localization

of GFP SM22α (Fig. 22B and C). The exchange of the h1CaP CH domain for the CH domain of Vav reveals a similar picture, and h1CaP-VavCH also failed to associate with actin filaments (Fig 22A and E).

These results support the concept that type 3 CH domains have distinct functions in different molecular species. Although they share a few common core residues that allow similar three dimensional structures, they are functionally diverse. The CH domain of Vav and SM22α appear unable to interact with the C-terminal tail of h1CaP, and hence, to alleviate the block on the actin-binding activity in h1CaP. Interestingly, the predicted residues of h1CaP involved in the binding of the tail (Arg44, Arg45 and Asn49, see section 3.3) are not present in SM22α, and only Arg45 is present in Vav (Fig. 21).

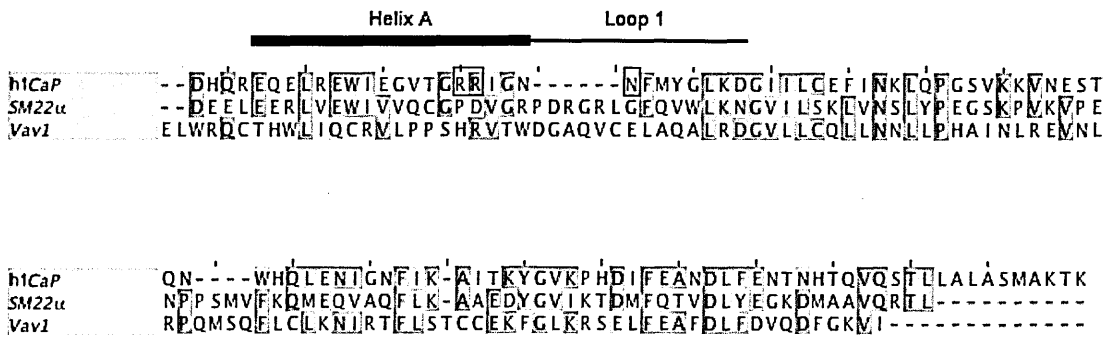
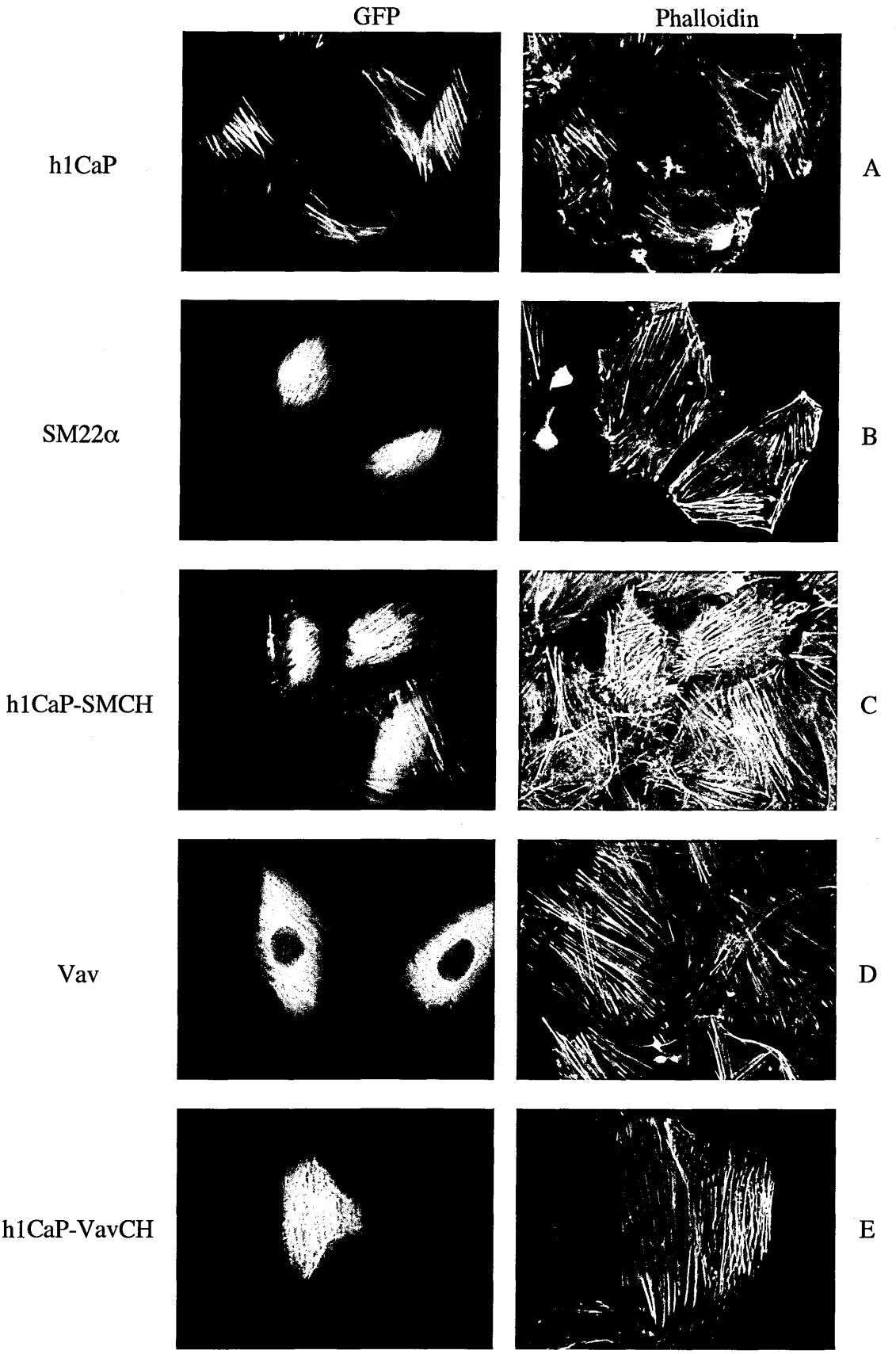


Figure 21. Alignment of the CH domain sequences of h1CaP (CaP), SM22α and Vav1. Conserved residues are highlighted in blue and the h1CaP residues involved in the interaction with the C-terminal tail are marked with black squares.

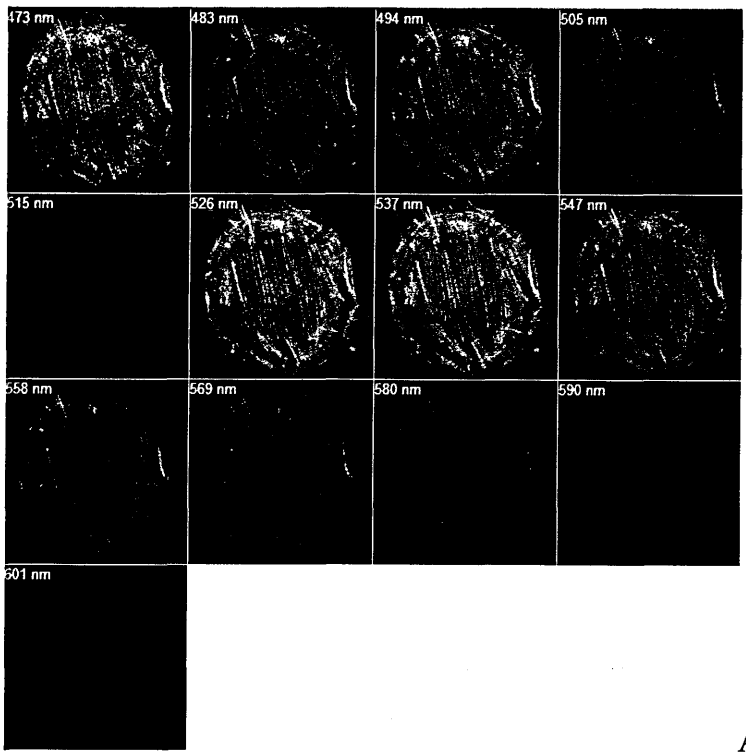
Figure 22. (page 89) Subcellular localization of SM22α, Vav1 and h1CaP constructs. (A) H1CaP is mainly found on stress fibres of cells. (B) SM22α localizes mainly in the cytoplasm of cells and associates poorly with stress fibres in formaldehyde-fixed cells. (C) H1CaP carrying the CH domain of SM22α is found in the cytoplasm as well on stress fibres of the cells. (D) Vav1 is found in the cytoplasm as h1CaP carrying the CH domain of Vav (E).

Figure 22

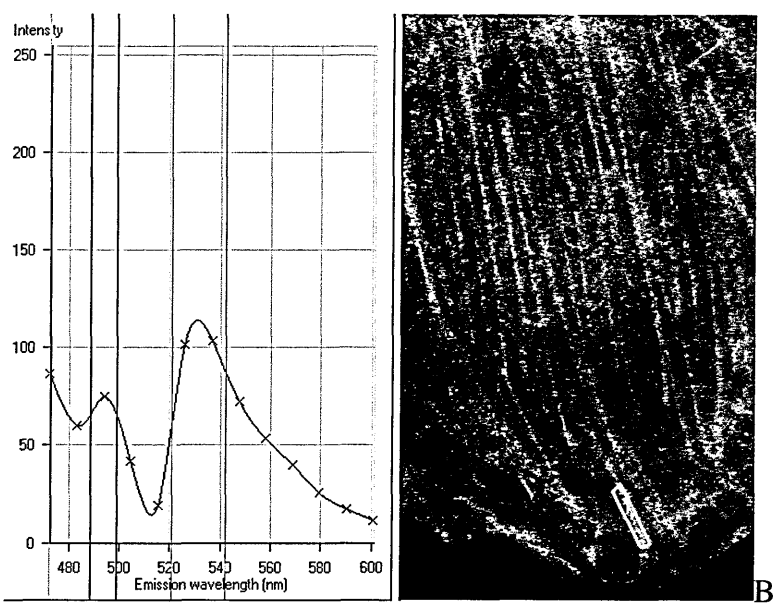

GFP
Phalloidin
h1CaP
A
SM22α
B
h1CaP-SMCH
C
Vav
D
h1CaP-VavCH
E

3.7 Molecular proximity of the CH domain and the tail of h1CaP

The data presented thus far indicate that the CH domain and the tail of h1CaP interact *in vitro*. However, it is important to analyze whether this interaction can also occur in live cells. Thus, fluorescence resonance energy transfer (FRET) was used to analyze whether the CH domain and the tail are in close enough proximity to allow a physical interaction. For this purpose, h1CaP was cloned into the pCDNA mammalian expression vector and fused to a citrine (YFP) and cyan fluorescence protein (CFP) at the N- and C- terminus, respectively (Y-h1CaP-C). A7r5 cells were transfected and FRET was measured under a Zeiss LSM510 META confocal microscope, using the donor dequenching technique (Bastiaens and Jovin, 1996; Szaba et al., 1992). This technique is based on the fluorescence recovery of the donor after acceptor bleaching. When the CFP of Y-h1CaP-C is excited two emission peaks are observed, one at 490 nm, corresponding to the CFP emission, and a second at 530 nm, corresponding to the emission of the YFP (Fig. 23A and B). The intensity of the second peak suggests that energy transfer occurs between the CFP and YFP. Indeed, the intensity of the CFP is seen to increase when the YFP is bleached, suggesting that part of the CFP energy was transferred onto the YFP (Fig. 24A). This effect is not observed in Y-h1CaPΔT-C where the tail is not present (Fig. 24B) indicating that the N-terminal CH domain is close to the C-terminus of the h1CaP only in the presence of the acidic tail (Fig. 24C). The data obtained from measuring the CFP-fluorescence changes of Y-h1CaP-C and Y-h1CaPΔT-C was analyzed with the statistical student's τ-test, which resulted in a p-value of 0.045.



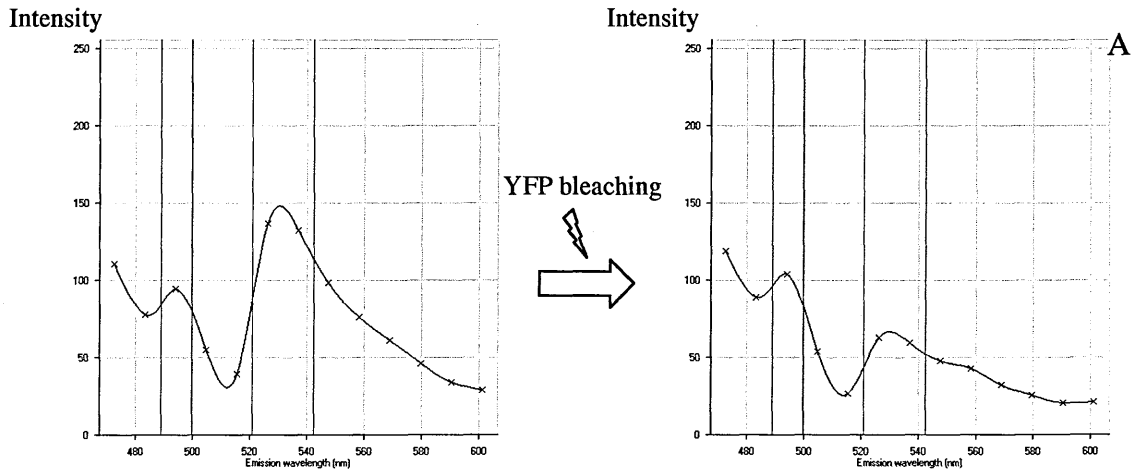
A

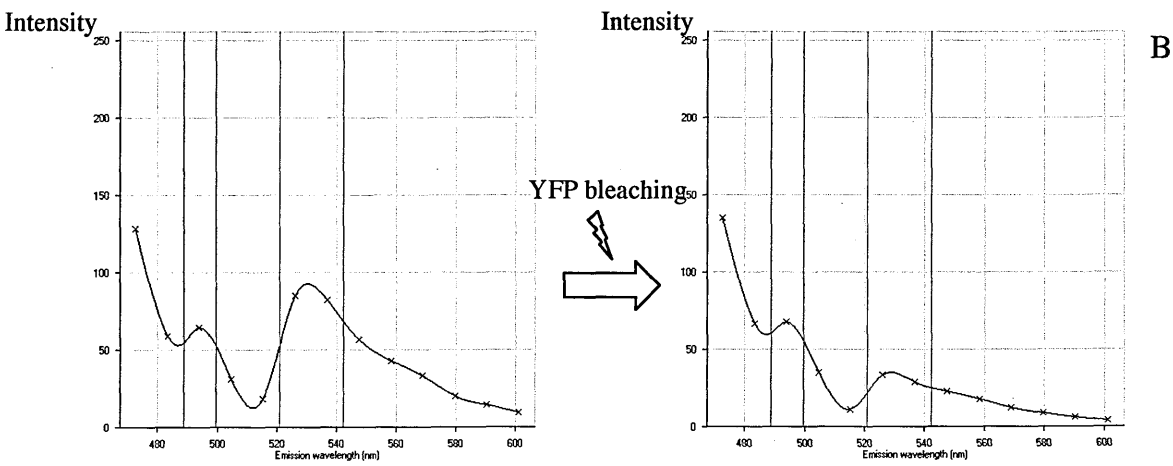


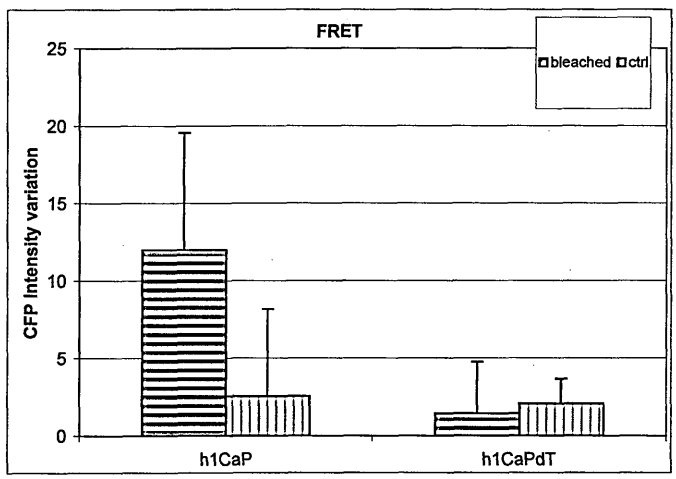
B

Figure. 23. Subcellular localization and CFP excitation of the h1CaP FRET sensor. (A) Excitation of CFP produces two peaks of excitation, one between 473 nm and 494 nm and a second peak between 526 nm and 537 nm. (B) Excitation spectra of h1CaP upon CFP excitation in the region of interest (ROI). The ROI is marked with a red box.

Figure 24


Intensity
Intensity
A
YFP bleaching
Emission wavelength (nm)
Emission wavelength (nm)


Intensity
Intensity
B
YFP bleaching
Emission wavelength (nm)
Emission wavelength (nm)


FRET
bleached
ctrl
CFP Intensity variation
h1CaP
h1CaPdT

C

Figure 24. (page 92) Energy transfer of h1CaP constructs carrying and N-terminal YFP and a C-terminal CFP. (A) Bleaching the YFP of h1CaP induces an increment in the fluorescence intensity of the CFP. (B) Bleaching the YFP of h1CaP lacking the C-terminal tail does not increase the fluorescence of the CFP. (C) Measurement of the relative changes in the fluorescence intensity of the CFP after bleaching the YFP. The control is the change of fluorescence intensity of CFP in a non-bleached area. Student's τ-test with one tail and with unequal variance gave a statistical p-value of 0.045.

3.8 The C-terminal tail controls the actin-binding dynamic of h1CaP

It has previously been shown that the C-terminal tail of h1CaP negatively regulates the h1CaP actin-binding activity (Burgstaller et al., 2002). A direct approach to analyze protein dynamics in cells is the use of fluorescence recovery after photobleaching (FRAP). FRAP utilizes the phenomenon of photobleaching of fluorescent probes to measure molecular mobility. Thus, A7r5 cells were transfected with the GFP tagged probes of h1CaP, h1CaPΔT, h1CaPΔCHD and h1CaPΔCHDΔT, to study their cellular dynamics. FRAP was performed under a Zeiss LSM510 confocal microscope, and the region of interest was bleached using 100% intensity of the 488 nm laser. Images were acquired before and after bleaching and a third image was taken after 2 or 5 minutes to avoid excessive bleaching of the sample. The results show a relatively high stability for full-length h1CaP. While GFP can recover 90% of the fluorescence 10 seconds after the bleaching time (Goodwin and Kenworthy, 2005; Swaminathan et al., 1997), the fluorescence recovery of h1CaP after bleaching was approximately 40% in 5 minutes (Fig. 25A). This slow recovery rate was even more pronounced in h1CaP lacking the tail (h1CaPΔT) (Fig. 25A), suggesting a slower turnover for this construct on actin filaments, as was proposed earlier (Burgstaller et al., 2002). On the other hand, the fluorescence of h1CaPΔCHD and h1CaPΔCHDΔT was rapidly recovered (Fig. 25B), and after 2 minutes of the bleaching time, 80% and 90% of the fluorescence was recovered, respectively. These results strongly suggest

that both the CH domain and the tail are involved in the regulation of the actin-binding activity of h1CaP. In addition, the h1CaPΔCHDΔT construct was expected to have recovery rate similar to h1CaP, which it doesn't (Fig. 25A and B). The fast fluorescence recovery observed with the h1CaPΔCHDΔT could be due to an impairment of its regulatory mechanism.

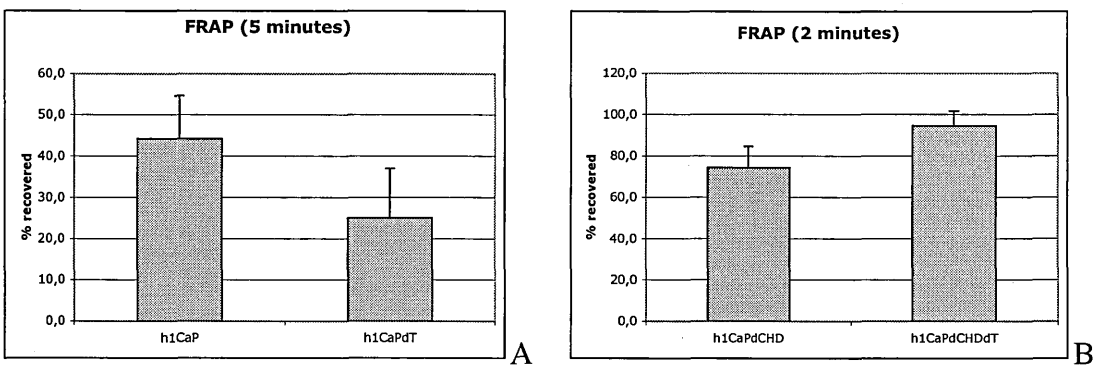


Figure 25. Fluorescence recovery after bleaching of GFP-tagged h1CaP constructs. (A) Recovery of h1CaP is 45% after 5 minutes. The recovery of h1CaP lacking the tail is 25%. (B) H1CaP lacking the CH domain or the CH domain and the tail shows faster recoveries. After 2 minutes more than 70% of the fluorescence of h1CaPΔCHD was recovered, and almost 90% for h1CaPΔCHDΔT.

CHAPTER 4: RESULTS II

4.1 Calponins localize at the ingression furrow and the contractile ring

Two prominent actin-rich structures are formed during mammalian cytokinesis, namely the cleavage furrow and the contractile ring. These two structures contain myosin II, which facilitates furrow ingression and the constriction of the plasma membrane of cells, which is necessary to separate the mother cell into two daughter cells. During metaphase, cells round up and at the beginning of anaphase cells elongate, forming a cylindrical shape, and a furrow begins to ingress in the same plane where the contractile ring is localized (Robinson and Spudich, 2004). Although actin and myosin II are key players in the formation and function of these structures, the presence of ABPs is also important (Reichl et al., 2008). These allow propagation of the tension generated by myosin into the crosslinked actin network and to the plasma membrane.

H1CaP was discovered in an attempt to identify troponin related proteins in smooth muscle, and most experiments have focused on its actin-binding activity and regulation of smooth muscle contraction. It was shown that overexpression of h1CaP can reduce cell proliferation and tumorigenicity of several cell lines (Lener et al., 2004; Ogura et al., 2006). Furthermore, an increment of binucleated cells is observed in cells transfected with h1CaP suggesting a role of h1CaP during cell division (Lener et al., 2004). Since h1CaP is an ABP and inhibits the actin-activated ATPase activity of myosin II it could also play an important role during cytokinesis. Therefore I have used an antibody against h1CaP to detect the endogenous protein, and transfected A7r5 cells with the three calponin isoforms tagged with GFP to monitor their localization during mitosis. H1CaP (Fig 26A, B and C), h2CaP (Fig. 27A) and h3CaP

(Fig. 27B) all localized to the ingression furrow. Before the ingression of the furrow, these CaPs accumulate at the cell periphery marking the area where the ingression of the furrow starts. Confocal images and thre dimensional projections of confocal images show the localization of endogenous h1CaP with the contractile ring (Fig. 28).

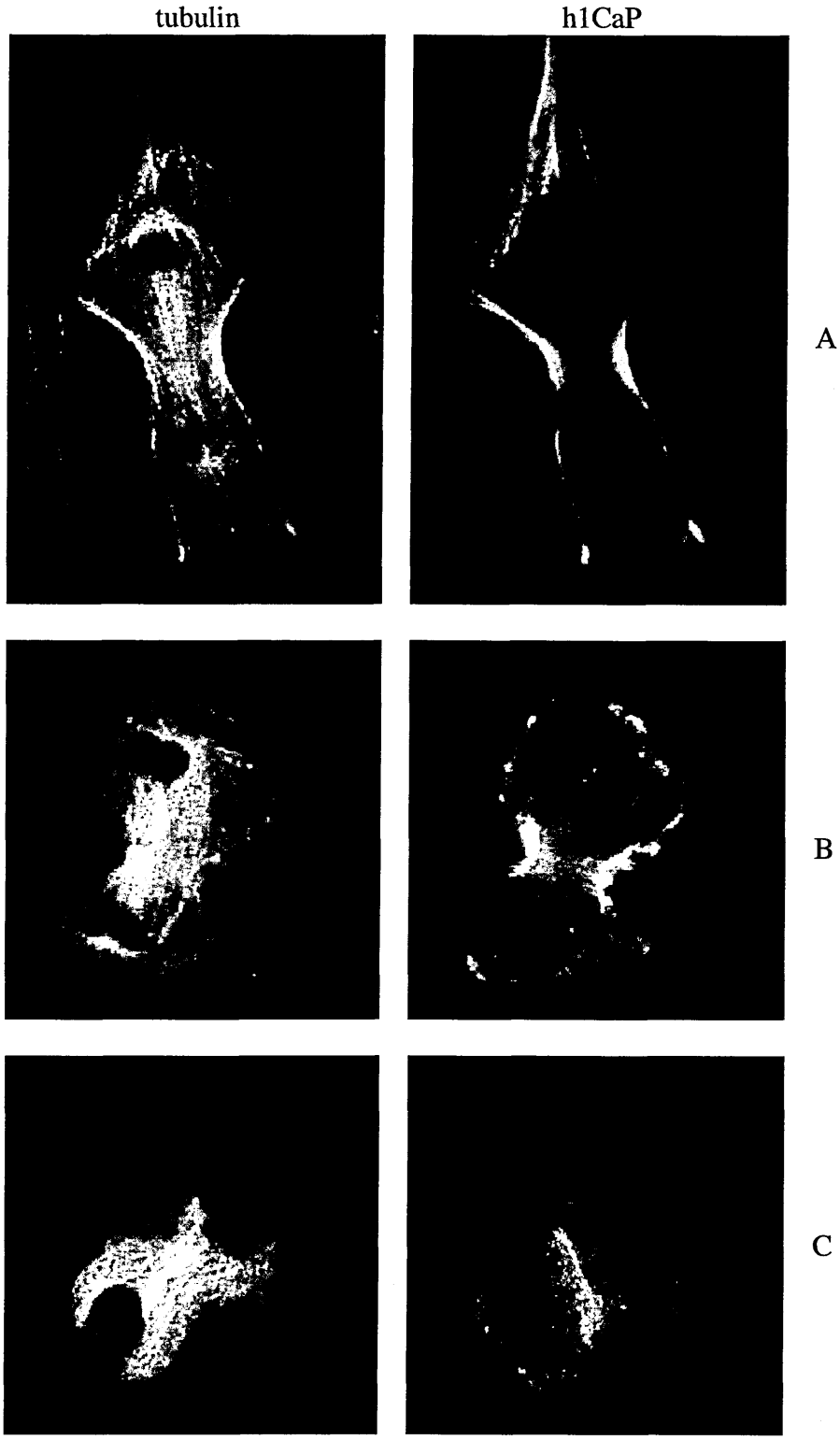


Figure 26. Subcellular localization of endogenous h1CaP and microtubules in A7r5 during cytokinesis. (A) H1CaP localizes at the ingression furrow at both sides of the cell equator. (B) H1CaP localizes at the ingression furrow and the contractile ring. (C) H1CaP is found in the contractile ring.

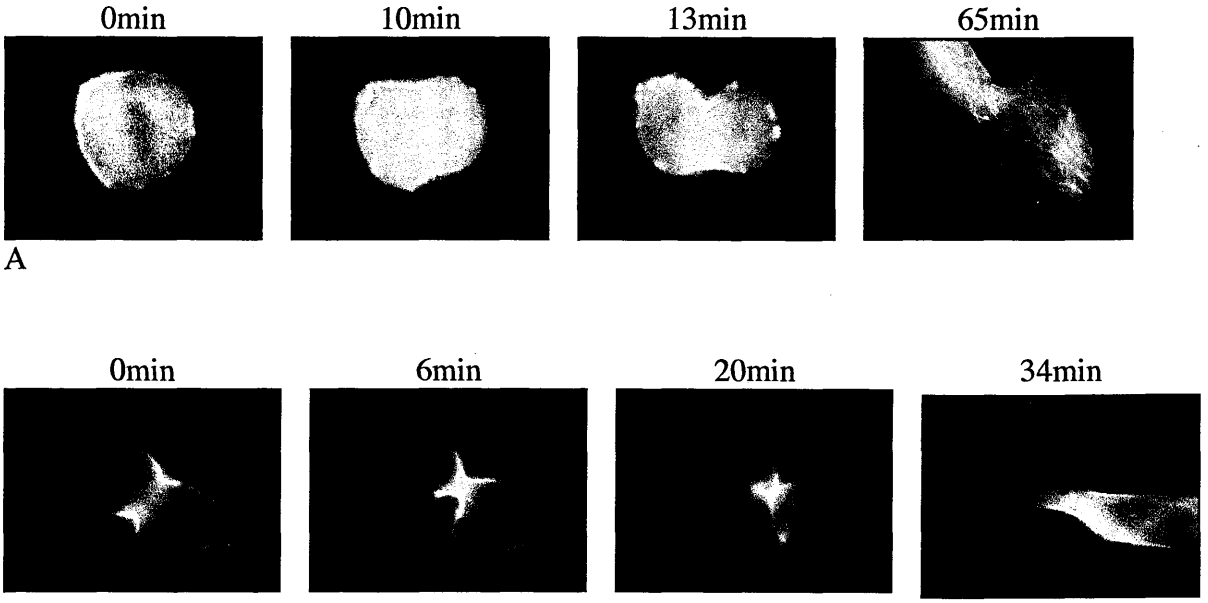


Figure 27. Subcellular localization of GFP tagged (A) h2CaP and (B) h3CaP in live A7r5 cells. Both proteins localize at the ingression furrow during anaphase.

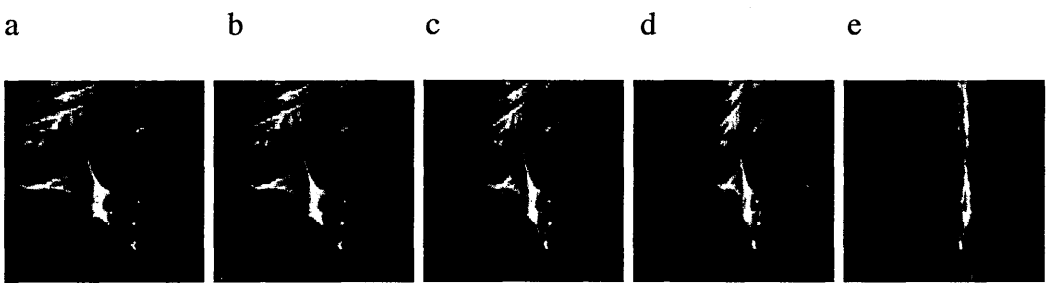
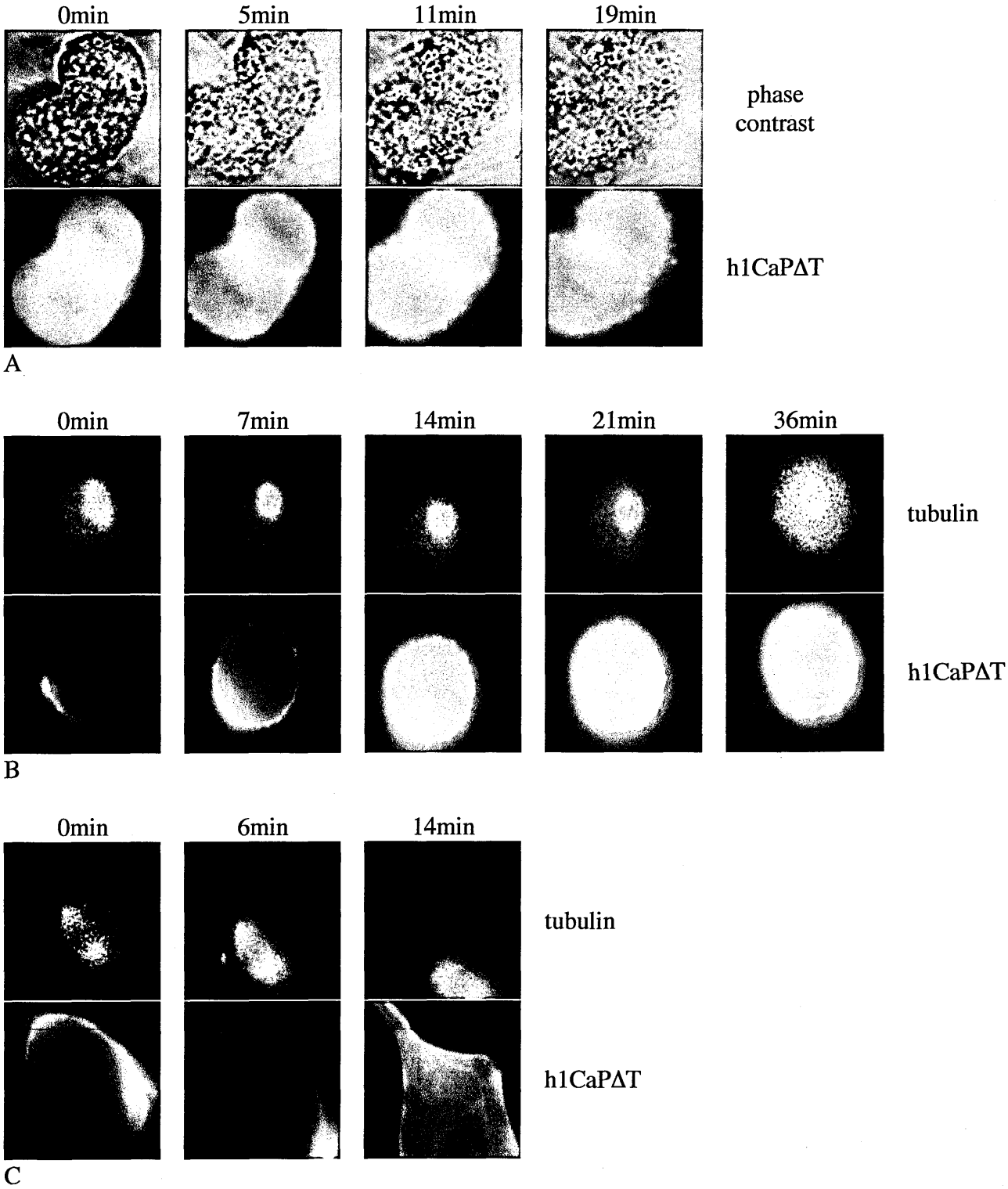


Figure 28. Three dimensional projection of confocal images of endogenous h1CaP. H1CaP is observed in the contractile ring (a to e).

4.2 Overexpression of h1CaP mutants induce defects in cell division

The finding that h1CaP localizes at the ingression furrow and the contractile ring raises several questions. Does h1CaP regulate cell division? If so, which phase and how is it regulated? As shown before, phosphorylation might not be relevant for the actin-binding function of h1CaP in interphase cells, however, it could be relevant during cell division. Furthermore, the CH domain and the tail, which can regulate h1CaP dynamics, could also play a role during cell division. Indeed, overexpression of h1CaP lacking the C-terminal tail and tagged with GFP (h1CaPΔT) induced defects in cells division (Fig. 29). This cell division impairment ranges from a block of ingression furrow formation and progression (Fig 29A and B) to impairment of abscission (Fig 29C and D) and mislocalization of the ingression furrow (Fig 29E). A block in metaphase is observed in cells overexpressing h1CaP-S175/254A tagged with GFP in A7r5 cells (Fig. 30A), while cell division progresses normally in cells overexpressing h1CaP-S175/254D, which mimics serine phosphorylation (Fig. 30B).

Figure 29


0min
5min
11min
19min
phase contrast
h1CaPΔT
A
0min
7min
14min
21min
36min
tubulin
h1CaPΔT
B
0min
6min
14min
tubulin
h1CaPΔT
C

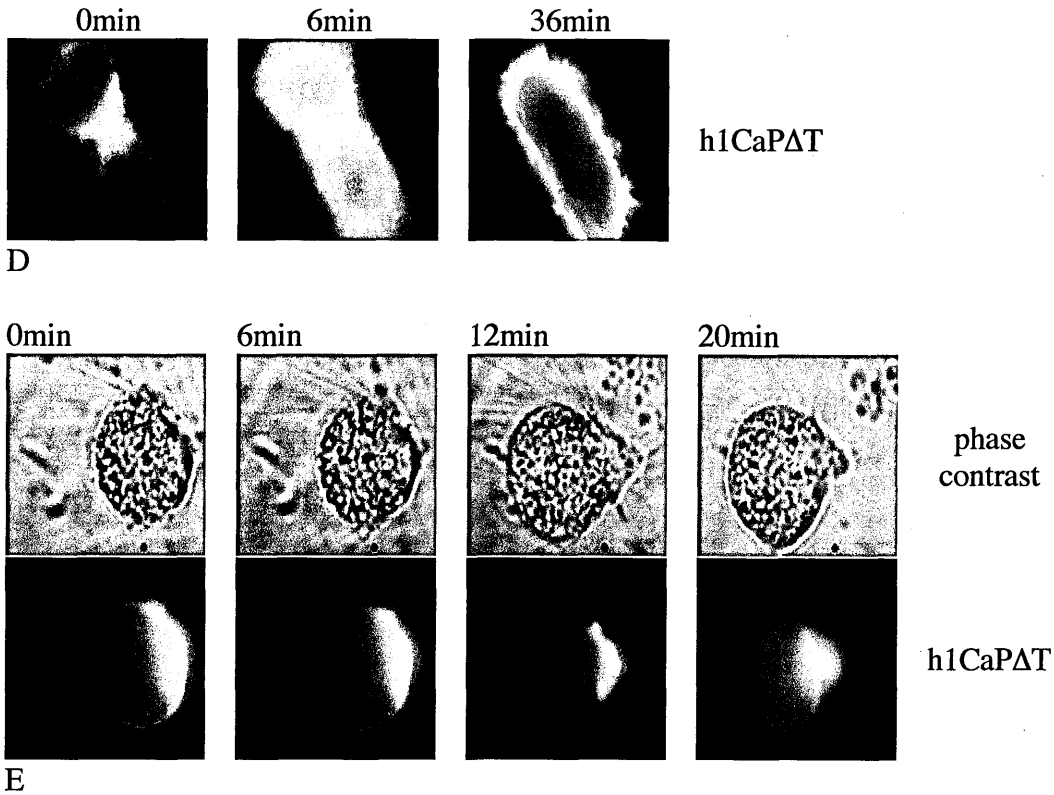


Figure 29 (pages 100 – 101). Subcellular localization and effects of tail-deleted h1CaP during cell division. (A) H1CaPΔT localizes at the ingression furrow, but the furrow does not progress. (B) H1CaPΔT localizes at the cell periphery during metaphase but there is no formation of the furrow and anaphase does not initiate. (C) H1CaPΔT localizes at the cell periphery during metaphase. A cell in interphase is observed without going through the anaphase. (D) H1CaP localizes to the ingression furrow. Despite a normal anaphase, the two daughter cells do not separate. (E) H1CaP localizes to only one side of the equator without the formation of the ingression furrow.

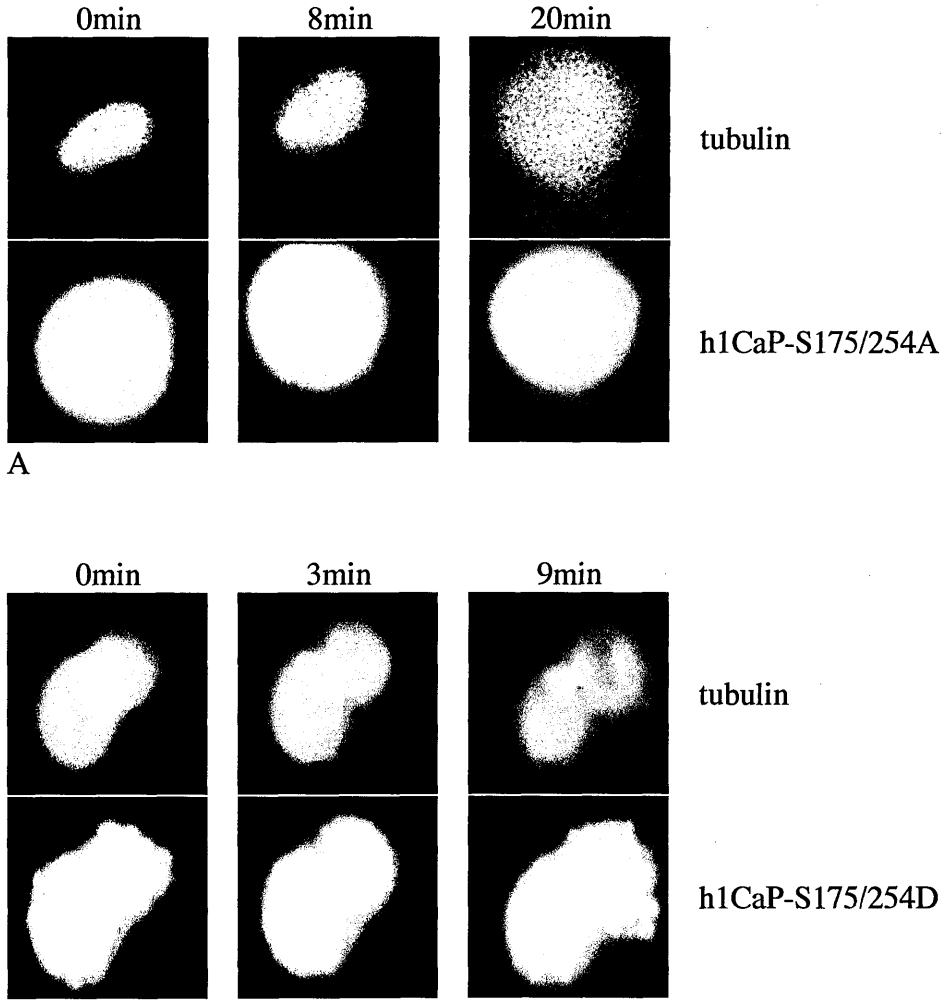


Figure 30. Subcellular localization and effects of h1CaP Ser175 and 254 mutants in live A7r5 cells. (A) CaP-S175/254A is observed in the cytoplasm and cell division does not progress. (B) CaP-S175/254D is observed in the cytoplasm and cell division progresses normally.

CHAPTER 5: DISCUSSION

The actin cytoskeleton is regulated by ABPs, which are involved in the dynamic assembly and disassembly of actin filaments. ABPs regulate actin dynamics by promoting actin polymerization and the severing of actin filaments, sequestering actin monomers, or crosslinking and stabilizing actin filaments into networks and bundles. The actin cytoskeleton is under constant regulation in many physiological and pathological situations, such as cell division, locomotion and cell differentiation. Many human disease conditions such as cancer, chronic inflammation and angiogenesis are characterized by an increase in cell locomotion, where cells escape from a tissue, reach and cross the endothelial cell barriers of the blood vessels, and infiltrate other tissues. All these steps require constant adaptations of the actin cytoskeleton. Thus, defining the mechanisms that underlie the actin-binding activities of ABPs will lead to a better understanding of how these proteins work and of their involvement in human diseases. In this study an attempt was made to describe the molecular mechanism that regulates the actin-binding activity of h1CaP.

5.1 H1CaP phosphorylation

In vitro phosphorylated h1CaP cannot associate with actin filaments (Winder and Walsh, 1990b). This finding suggests that cells exploit this type of post-translational modification to regulate the actin-binding activity of h1CaP. H1CaP can be phosphorylated by PKC (Winder and Walsh, 1990b), ROCK (Kaneko et al., 2000), Ca^{2+}/CaMKII (Winder and Walsh, 1990a) and Fyn (Abouzaglou et al., 2004), however it is not known if these processes also occur *in vivo*. In addition, the phosphorylation sites are not accessible when CaP is associated with actin filaments (Nagumo et al., 1994). Thus other mechanism(s) must regulate the interaction of

h1CaP with F-actin. I have first analyzed the role of serine 175 phosphorylation in cells, which has been described as the main phosphorylated residue in h1CaP (Winder et al., 1993a). It was previously shown that the S175A mutation weakens the binding of h1CaP with actin filaments *in vitro* implying a crucial role of the hydroxyl side chain in position 175 in the interaction of h1CaP with actin (Tang et al., 1996; Uyama et al., 1996). In the same study, a S175T mutant did not affect the binding activity of h1CaP, confirming the importance of a phosphorylatable hydroxyl side chain in the binding activity of h1CaP. Even more, a S175D mutant showed very similar effects to those of Ser175A supporting the hypothesis that a hydroxyl group at position 175 is necessary for h1CaP to bind actin filaments and that phosphorylation inhibits the binding activity. However substitution of Ser175 for an aspartate or alanine in fixed cells (shown in this study) and live cells (data shown) did not affect the ability of h1CaP to associate with actin thin filaments. Therefore, the presence of Ser175 might not be as crucial in cells as it is *in vitro* since the dramatic effects on the actin-binding activity of h1CaP *in vitro* is not observed in cells. In contrast, the double mutation of Ser175 and Ser254 resulted in low levels of colocalization with actin filaments regardless of the presence of an alanine or aspartate. Based on the observations of Tang et al. 1996, two scenarios could explain these results: in the first scenario the hydroxyl groups of Ser175 and Ser254 would be the major contributors for the h1CaP binding with actin filaments. In the second scenario, Ser254, and not Ser175, would be the critical residue for the actin-binding activity of h1CaP. The first scenario can be questioned because in the present study, it is shown that mutations of Ser175 do not affect the actin-binding function of h1CaP in cells. However, earlier experimental data showed that phosphorylation of Ser175 changed h1CaP conformation (Jin et al.,

2000), which could then expose Ser254 to kinases, and lead to a reduction in the affinity of h1CaP for actin filaments.

It was found that Tyr182 and Tyr261 of h1CaP are phosphorylated *in vitro* by the Src family kinase Fyn, and that tyrosine phosphorylation significantly reduces the affinity of h1CaP for actin filaments (Abouzaglou et al., 2004). Therefore, I analyzed the cellular localization of h1CaP in cells incubated with $NaVO_4$, which did not differ from that in untreated cells. These data suggest that phosphorylation does not represent the major regulatory mechanism for h1CaP activity in cells. However, other h1CaP phosphorylation sites have been described, such as Thr184 (Nakamura et al., 1993), Ser71, Ser215 (Winder et al., 1993a), Thr 170, Thr180 and Thr 259 (Kaneko et al., 2000). Those residues are not as extensively phosphorylated as Ser175 and Ser254, thus they were not included in this analysis.

Both Ser175 and Ser254 of h1CaP are within conserved PKC-substrate consensus sequences (Kennelly and Krebs, 1991; Pearson and Kemp, 1991), which may explain the preference of PKC for these sites. However, the existence of a phosphorylatable consensus sequence in proteins does not ascertain that a protein is phosphorylated. Although h1CaP is efficiently phosphorylated *in vitro*, it is possible that *in vivo* h1CaP does not interact with PKC, or that the serines are not accessible to the kinase. Indeed, colocalization was not observed between h1CaP and PKCε in resting A7r5 cells (data not shown). Nevertheless, PKC might interact and phosphorylate h1CaP in diverse physiological situations *in vivo*.

5.2 Intramolecular interaction between the CH domain and the tail of h1CaP

The regulation of h1CaP at the molecular level is poorly understood. Regulation by phosphorylation is discussed above (see 5.1) and does not appear to be the major regulatory mechanism for the actin-binding activity of h1CaP. On the other hand, Ca^{2+} binding proteins such as CaM and S100 block the inhibitory function of h1CaP on the actin-activated ATPase activity of myosin *in vitro* (Kolakowski et al., 1995). In addition, it was shown that the CH domain of h1CaP binds to ERK, PKCε and PKCα (Leinweber et al., 2000; Menice et al., 1997). However, the physiological relevance of these interactions is not known.

One study attempted to formulate a regulatory mechanism based on the inhibition of the actin-binding activity by the C-terminal tail of CaPs (Burgstaller et al., 2002). Interestingly, the tail-deleted forms of h1CaP and h2CaP showed a higher affinity for actin filaments in a biochemical assay that measures the amount of protein in cellular fractions with or without actin filaments. Therefore, the C-terminal tail of CaPs was postulated to regulate their actin-binding activity either by interfering with the ABS or by interacting with other regulatory proteins. In the present study, I have analyzed several h1CaP mutants in order to shed more light on the contribution of the CH domain and C-terminal tail on the actin-binding activity of the protein. I have demonstrated by fluorescence microscopy analysis that the C-terminal tail of h1CaP indeed affects its association with actin-containing thin filaments, which is even more pronounced when the CH domain is also missing. Therefore, the presence of the CH domain is able to suppress the actin-binding inhibitory function of the C-terminal tail. Indeed, I have shown that the CH domain binds to the C-terminal tail in *in-vitro* overlay assays. One can thus propose that the CH domain and the tail of h1CaP are the major functional modules that are responsible for the regulation of the actin-

binding activity of the protein. The interaction between these domains *in vivo* could influence the dynamics of the protein and modulate the strength of its binding to actin filaments. I have also shown by FRET analyses that the CH domain and the tail of h1CaP are in close proximity, and that the CH domain and the C-terminal tail of h1CaP regulate the protein dynamics in live cells. H1CaP and its tail-deleted form have slow fluorescence recovery times, suggesting that they are tightly associated with actin filaments. In contrast, h1CaP mutants lacking either the CH domain alone or both the CH domain and the tail show faster fluorescence recovery, which indirectly points towards a reduced affinity for actin filaments.

5.3 A hypothetical model for the molecular mechanism that regulates h1CaP-actin interaction

Considering the results of this study together with the conclusions of Burgstaller et al. 2002, a more detailed picture of the regulatory mechanism of the actin-binding activity of h1CaP is starting to emerge. Two flexible domains, the C-terminal tail and the N-terminal CH domain together appear to regulate h1CaP-actin binding. The actin-binding activity of h1CaP could be induced by an interaction between both of these terminal domains, which exposes the actin-binding interface generated by the triple CLIK[23] repeats. When the intramolecular contact between the CH domain and the tail is released (by a yet to be defined mechanism), the tail interacts with the CLIK[23] repeats and interferes with the actin-binding activity (Fig. 31). This mechanism requires to some extent a flexible protein that can shift between two conformations. Indeed, molecular flexibility has been observed in electron microscopy images of h1CaP (Stafford et al., 1995). Even more, the CLIK[23] repeats follow the consensus of a type I intrinsically unstructured protein (IUP)

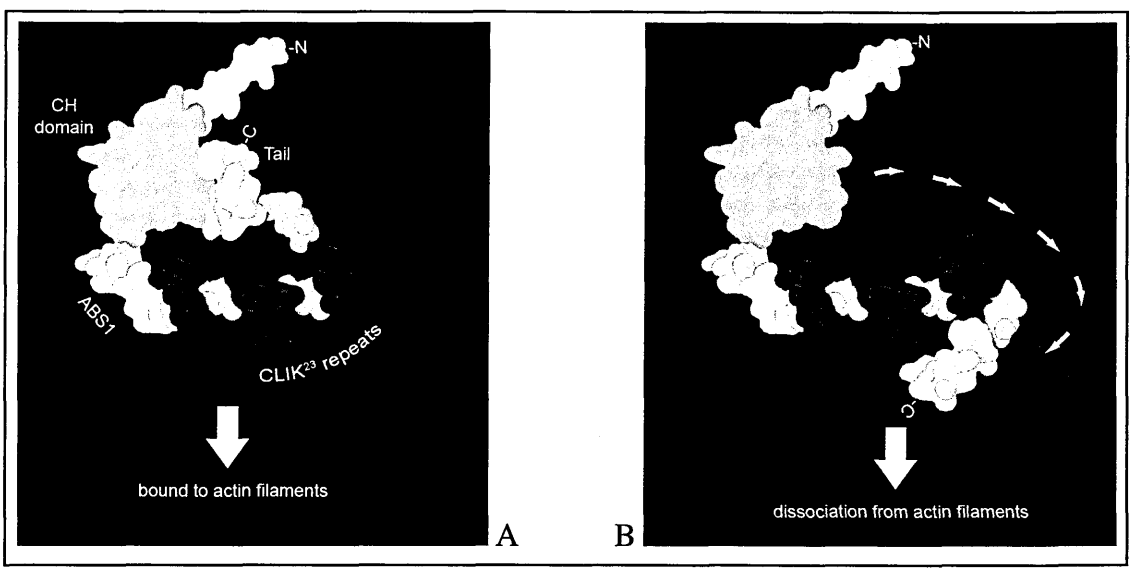


Figure 31. Hypothetical model of the two molecular states of h1CaP. (A) The C-terminal tail (cyan) of h1CaP interacts with the CH domain (green) exposing the $CLIK^{23}$ repeats (the repeats are in blue and intervening sequences in red) to the actin filaments. (B) Factors such as calcium binding proteins bind to the N-terminus of h1CaP (yellow) and displace the tail from the binding site of the CH domain. Once the tail is free it can interact with the $CLIK^{23}$ repeats, thus blocking the interaction with actin filaments.

(Czurylo et al., 2000; Stafford et al., 1995), suggesting a high propensity for protein flexibility. It is well established that IUPs often behave as flexible strings and that binding to a target molecule can induce structural rearrangements and the formation of physiologically relevant binding sites (Dyson and Wright, 2005).

Similar regulatory mechanisms that are mediated by the intramolecular interaction of two domains have also been described for other ABPs and proteins possessing type 3 CH domains. Vinculin is a 117-kDa protein that binds actin filaments and is present in focal adhesions (Geiger et al., 1980). The N-terminal head domain of vinculin binds to its C-terminal tail domain, which results in the masking of the actin-binding site (Johnson and Craig, 1995a). The vinculin tail binds acidic phospholipids that release the interaction with the head and expose the actin-binding sites (Johnson and Craig, 1995b). A similar regulatory mechanism has also been proposed for the actin-binding activity of the myristoylated alanine-rich C kinase substrate (MARCKS) protein and for the *Dyctyostelium discoideum* 34-kDa F-actin-bundling protein (Lim et al., 1999; Tapp et al., 2005). The GEF activity of Vav towards Rho and Rac GTPases is regulated by the intramolecular interaction between the N-terminal CH domain and the central ZF region of the protein (Zugaza et al., 2002). The binding of the CH domain to the ZF region results in the inhibition of the GEF activity. A similar mechanism was also proposed for the GEF ARHGEF6 or αPIX. A CH domain deleted form of ARHGEF6 is constitutively active, suggesting that the CH domain regulates the GEF activity of the protein (Daniels et al., 1999). These observations on Vav, ARHGEF6 and now h1CaP, suggest that the type 3 CH domains might have evolved as regulators of protein activities mediated through intramolecular interactions.

The CH domain – tail interaction may be regulated by several factors. It was shown *in vitro* that the Ca^{2+}-binding protein caltropin (S100A6) binds to the N-terminal region of h1CaP (Wills et al., 1994a) and inhibits the actin-binding activity of h1CaP (Wills et al., 1994b). In addition to caltropin, other Ca^{2+} binding proteins including CaM and other S100 family members, can bind to the N-terminal domain of h1CaP and block the inhibition of the actin-activated ATPase activity of myosin (Abe et al., 1990) and the removal of h1CaP from the actin filaments (Fujii et al., 1994). Considering these data and the results of the present study, it is probable that the interactions of Ca^{2+}-binding proteins with the N-terminal domain of h1CaP disrupts the interaction between the h1CaP CH domain and the tail and reduces its affinity for actin filaments. Furthermore, the N-terminal region of h1CaP where Ca^{2+}/CaM and caltropin bind, contains the binding area for the C-terminal tail (Mezgueldi et al., 1992; Wills et al., 1994a). PKCα, PKCε and ERK might also regulate the interaction between the h1CaP CH domain and the tail. The physiological relevance of these interactions, however, needs to be demonstrated (Leinweber et al., 2000; Menice et al., 1997). Nevertheless, the binding of Ca^{2+}/CaM and caltropin is intriguing, since they lack enzymatic (e.g. kinase) activity (Crivici and Ikura, 1995).

CaM is 15-kDa protein that is ubiquitously expressed in animals, plants, fungi and protozoa (Bazari and Clarke, 1981; Cheung, 1971; Eldik et al., 1980). It functions as a cytosolic Ca^{2+} receptor and binds four calcium ions, which induce a conformational change that enables Ca^{2+}/CaM to bind its target proteins (Crivici and Ikura, 1995; Krebs and Carafoli, 1982). It is possible that CaM and calcium fluxes regulate the actin-binding activity of h1CaP. Three-dimensional reconstructions of pure F-actin incubated with h1CaP or h1CaPΔCHD show that the $CLIK^{23}$ repeats are in contact and mediate the binding with the actin filaments, while the N-terminal

region comprising the CH domain is not (Galkin et al., 2006). Thus, it is possible that the N-terminal region remains accessible for the binding to the C-terminal tail. Furthermore, the N-terminal region of h1CaP could also interact with Ca^{2+}/CaM, which in turn would free the tail from the CH domain thus inhibiting actin binding. The C-terminal tail of h1CaP and Ca^{2+}/CaM might compete for binding to the same region of the h1CaP N-terminus. Furthermore, other Ca^{2+}-binding proteins such as caltropin and the smooth muscle calcium binding protein (SMCaBP) have significantly higher affinities for h1CaP than Ca^{2+}/CaM (Wills et al., 1993; Wills et al., 1994b); therefore, they might also regulate h1CaP activity in a similar manner.

As h1CaP only binds to calcium-bound CaM and not to the free form of CaM (Winder et al., 1993b), this suggests that intracellular calcium regulates the actin-binding activity of h1CaP.

5.4 Regulation of the actin-binding activity of calponin isoforms

All CaPs have a similar domain structure and their amino-acid sequences are highly conserved, except for their C-terminal tails, which are different in each isoform. The structural similarities suggest that CaPs share similar regulatory mechanisms in the cell. The isotype-specific acidic tail sequences might dictate the strength of the binding between the CH-domain and the tail, and the $CLIK^{23}$ repeats and the tail. It has previously been shown that the presence of the tail in h1CaP, h2CaP and h3CaP causes a reduction in the actin binding, and that the effects are more pronounced in h2CaP and h3CaP (Burgstaller et al., 2002; Danninger and Gimona, 2000). Notably, the isoelectric points (pIs) of the CaP tails vary considerably. The pI of h1CaP C-terminal tail is 5.88, whereas h2CaP and h3CaP tails have pIs of 3.09 and 3.53, respectively (Danninger and Gimona, 2000). These data

suggest that a more acidic tail probably reduces the affinities of CaPs for actin filaments. Indeed, almost 80% of the cellular h1CaP was retained in the cytoskeletal fractions of NIH 3T3 cells, while only 25% and 40% of the cellular h2CaP and h3CaP were retained (Danninger and Gimona, 2000). Thus, the pI of the h1CaP tail could account for less stringent regulation on the $CLIK^{23}$ repeats, and the electrostatic interactions between the tails and $CLIK^{23}$ repeats of h2CaP and h3CaP could be significantly higher, with the consequence of an even increased reduction in their actin-binding affinities compared to h1CaP. The *in-silico* modelling approach used to model the CH domain – tail interaction of h1CaP could also be applied to the other two isoforms but to date there are no homologous sequences in the data-bank of the National Center for Biotechnology Information (NCBI) that can be used as a template to provide a reliable three-dimensional structure for the tails of h2CaP and h3CaP. In addition, subtle differences are found at the amino-acid level between the CH domains of the CaPs, and the crystal structures of h2CaP and h3CaP CH domains have not yet been determined. These differences in the CH domains might account for an isoform specific mechanism of CH domain – tail interaction. Indeed, Arg44, Arg45 and Asn49 of h1CaP, which were predicted to be the major contributors to the stabilization of the CH domain and tail interaction, are not found in h2CaP, and only Asn49 is present in h3CaP. Therefore, it would be of great value to analyze the modes of interactions between the CH domains and the tails of these other two CaP isoforms.

Although CaPs might share similar regulatory mechanisms to modulate their actin-binding behaviour, they may well interact with different accessory proteins that control the interactions between their CH domains and C-terminal tails. It is known that h1CaP binds to TM, Ca^{2+}/CaM and S100, whereas h3CaP does not (Fujii et al., 2002; Mezgueldi et al., 1992; Vancompernolle et al., 1990; Wills et al., 1993). Thus,

considering the differences in the amino-acid sequences of CaPs and the different binding partners, one cannot rule out the possibility that the actin-binding activities of h2CaP and h3CaP are regulated in a different ways.

5.5 CaPs are involved in cytokinesis

In this study I have shown that CaPs not only bind to actin filaments during interphase but also throughout cell division, and overexpression of h1CaP mutants leads to aberrant cell division. The defects observed during cytokinesis in h1CaPΔT overexpressing cells may be due to a lack of control of h1CaP actin-binding dynamics, owing to the absence of the tail sequence. Indeed, FRAP experiments with transfected full-length h1CaP during ingression furrow formation show a faster fluorescence recovery compared to h1CaP during interphase suggesting the presence of a molecular mechanism that regulates the actin-binding kinetics. CaPs were found mainly in the ingression furrow and the contractile ring. These actin-based structures are contractile and many cytoskeletal proteins are associated with them, including actin-crosslinking proteins (e.g. cortexillin and α-actinin) and myosin II (Reichl et al., 2008). The contractile property of the ingression furrow and the contractile ring is what allows the constriction of the plasma membrane. H1CaP inhibits the actin-activated ATPase activity of myosin *in vitro* (Abe et al., 1990), therefore, during cytokinesis it might control the cyclic ATPase activity of myosin II. Furthermore, calcium-binding proteins could play a key role in controlling the inhibitory activity of h1CaP on the actin-activated ATPase activity of myosin (see 5.3). Ca^{2+}/CaM was shown to localize at the cleavage furrow (Li et al., 1999), and thus it could release h1CaP from actin filaments and alleviate the inhibitory activity of h1CaP that is exerted on myosin, while it activates MLCK (Pearson et al., 1988). CaM activation of

MLCK regulates the force-generating actomyosin ATPase activity that is necessary for the gliding of filaments during cytokinesis. Whether h2CaP and h3CaP might also regulate myosin II during cell division remains to be determined in detail. It is still largely unknown whether h2CaP can inhibit the ATPase activity of myosin and, h3CaP has little effect on the myosin activity (Fujii et al., 2002). This might be due to the presence of a short ABS (spanning residues V142 to I163) in h1CaP, which is non-functional in h2CaP, and was shown to be responsible for the inhibition of the ATPase activity of myosin II (Mezgueldi et al., 1995).

A role of calponins in cell division is consistent with previous results. It was shown before that overexpression of h1CaP in cultured smooth muscles cells and fibroblasts suppresses their proliferation (Jiang et al., 1997). Also, h1CaP-deficient mice display increased bone formation, although it cannot be excluded that this increment in cell number is due to a increment in the expression levels of the bone morphogenetic protein (BMP), which is a potent initiator of cartilage and bone formation (Yoshikawa et al., 1998). In addition, during smooth muscle injury, quiescent SMCs change their phenotype and become proliferative at the same time as h1CaP expression levels are reduced (Samaha et al., 1996). More recently, it was shown that TNF-α and PDGF-BB, which are potent inducers of cell proliferation, are able to reduce the expression levels of h1CaP (Sugenoya et al., 2002). All these data imply a role for h1CaP as a suppressor of cell proliferation and underscore the necessity for cells to control the expression/activation levels of h1CaP during cell division.

Although phosphorylation of h1CaP does not seem to play a key role in cells in interphase, it could be important during cytokinesis. In this study was shown that h1CaP-S175/254A mutation deregulate cell division while division progress normally

with the h1CaP-S175/254D mutants. However, the cellular localization of these two mutants in dividing cells differs significantly compared to the wild-type h1CaP. During cell division, h1CaP localizes to the cell periphery and the contractile ring, whereas both serine mutants are found in the whole cell, possible due to the necessity of the hydroxyl groups of the serines for actin binding activity of h1CaP (see section 5.1). The function of h1CaP during cytokinesis might be controlled by ROCK, that is able to phosphorylate h1CaP *in vitro*, and which localizes at the contractile ring and regulates cytokinesis (Kosako et al., 1999; Yasui et al., 1998); hence ROCK could also act on h1CaP to control its actin-binding activity and thus its influence on cell division. Interestingly, using a phospho-motif finder (Amanchy et al., 2007) I have detected several motifs in the h1CaP sequence that may be recognized by a variety of signalling molecules such as STAT3, FRIP, Crk, RasGAP, shc and Grb, and it will be of interest to determine whether h1CaP interacts with these components *in vitro* and *in vivo*. Such efforts could lead to the identification of the signalling pathway(s) that regulate h1CaP function and activity.

ABBREVIATIONS

ABD	actin-binding domain
ABP	actin-binding protein
ABS	actin-binding site
Ac	acidic region
ADF	actin depolymerization factor
ADP	adenosine diphosphate
APBS	adaptive Poisson-Boltzman solver
ARE	actin responsive element
ARHGEF	Rho guanine nucleotide exchange factor
Arp	actin related protein
Arpc	actin related protein complex
ATP	adenosine triphosphate
BMP	bone morphogenic protein
CAD	caldesmon
CaM	calmodulin
CaMKII	calmodulin kinase II
CaP	calponin
CaPZ	capping protein Z
Cdc42	cell division cycle 42
CFP	cyan fluorescent protein
CH	calponin-homology
CLIK[23]	calponin-like repeats
DH	Dbl homology
DMEM	Dulbecco's Modified Eagles Medium
DNA	deoxyribonucleic acid
Dp	dystrophin protein
EDTA	ethylenediamine tetraacetic acid
eMLC	essential myosin light chain
F-actin	filament actin
FLN	filamin
FRAP	fluorescence recovery after photobleaching
FRET	fluorescence resonance energy transfer
G-actin	globular actin
GAP	GTPase-activating protein
GBD	GTPase-binding domain
GDI	guanine nucleotide dissociation inhibitors
GEF	guanine exchange factor
GFP	green fluorescent protein
GST	glutathione-S-transferase
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
h1CaP	basic calponin
h2CaP	neutral calponin
h3CaP	acidic calponin
HEPES	4-(2-hydroxy-ethyl)-piperazine-1-ethane-sulfonic acid
IL	interleukin
IPTG	isopropyl-β-D-1-thiogalactopyranoside
IUP	intrinsically unstructured protein

-kDa	kilo Dalton
LIMK	LIM kinase
MAPK	mitogen-activated protein kinase
MARCKS	myristoylated alanine-rich C kinase substrate
mDia	mammalian diaphanous
MHC	myosin heavy chain
MLC	myosin light chain
MLCK	myosin light chain kinase
MTOC	microtuble-organizing center
NCBI	National Center for Biotechnology Information
NF	neurofilament
N-WASp	neuronal WASp
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PH	pleckstrin-homology
PI	phosphatidylinositol
pI	isoelectric point
PIP	phosphatidylinositol phosphate
PIP_2	phosphatidylinositol (4,5)-bisphosphate
PIP_3	phosphatidylinositol (3,4,5)-triphosphate
PKC	protein kinase C
PMSF	phenyl-methylsulphonyl fluoride
Rac	Ras-related C3 botulinum toxin substrate
Rho	Ras homology
rMLC	regulatory myosin light chain
ROCK	Rho kinase
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SH3	src homology domain 3
SM22	smooth muscle protein 22
SMC	smooth muscle cell
SMCaBP	smooth muscle calcium binding protein
TGF	transforming growth factor
TGN	transgelin
TM	tropomyosin
Tmod	tropomodulin
Tn	troponin
TNF	tumor necrosis factor
VCA	verproline-cofilin-homology-acidic
WASp	Wiskot-Aldrich-syndrome protein
WH	WASp homology
YFP	yellow fluorescent protein

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